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ASPECTS OF PHOSPHATE UTILIZATION BY BLUE-GREEN ALGAE

by

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FOREWORD

Effective regulatory and enforcement actions by the Environmental Protection Agency would be virtually impossible without sound scientific data on pollutants and their impact on environmental stability and human health. Responsibility for building this data base has been assigned to EPA's Office of Research and Development and its 15 major field installations, one of which is the Corvallis Environmental Research Laboratory (CERL).

The primary mission of the Corvallis Laboratory is research on the effects of environmental pollutants on terrestrial, freshwater, and marine ecosystems; the behavior, effects and control of pollutants in lake systems; and the development of predictive models on the movement of pollutants in the biosphere.

This report is an attempt to define the basic physiological phosphate kinetics in blue-green algae. As such it is an important beginning towards the understanding of phosphorus dynamics in the aquatic environment.

A. F. Bartsch
Director, CERL

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SECTION I

INTRODUCTION

A paradox exists in the apparent success of blue-green algae in eutrophic waters. Blooms of these species often occur when the supply of nutrients is at its lowest point (Pearsall, 1932). Hutchinson (1973) suggests that a mechanism exists in these organisms which makes them extremely efficient at taking up phosphorus at very low concentrations. This suggestion is supported by evidence from Mackereth (1953), Rigler (1956), Stewart and Alexander (1971), and Lean (1973). The mechanism suggested by Hutchinson is most likely a physiological condition referred to by other workers as "Phosphat-Uberkompensation" (Liss and Langen, 1962), "Polyphosphate Overplus Phenomenon" (Harold, 1964, 1965; Harold and Harold, 1963; Voelz *et al*, 1966), or "rapid uptake" (Shapiro, 1967) and provides for a restoration of phosphorus supply following phosphate starvation or phosphate limitation. The specific mechanism involved results in the formation of polyphosphates, long chain, osmotically inert polymers of inorganic phosphate.

The biochemical nature of polyphosphates, their periodicity and role in phosphorus metabolism in the cell, and the significance of their presence in algae common in eutrophic waters require further examination. Difficulties arise when one attempts to assess the extensive literature available on phosphate metabolism. Both environmental and physiological parameters of the uptake phenomenon described in the literature vary. In addition, no exhaustive study has been conducted on one test organism.

Consideration of these factors led to studies which were designed to determine if the polyphosphate overplus phenomenon exists in a blue-green alga, *Plectonema boryanum* Gomont. The experiments are an attempt to elucidate the effect of the environmental parameters on the physiological and cytological responses of the alga to conditions of both phosphate limitation and excess available phosphate.

SECTION II

SUMMARY

The effects of various external phosphate concentrations on physiological and cytological aspects of *Plectonema boryanum* have been studied. *P. boryanum* was found to tolerate a wide range of phosphate concentrations, from 1 to 1000 mg of phosphate per liter. Growth of the alga in these concentrations was characterized by changes in the subcellular distribution of phosphorus-containing compounds and in ultrastructural changes which were monitored by transmission electron microscopy.

Culturing the alga in phosphate-free or phosphate deficient medium led to general reductions of phosphate in all cell fractions examined, with the most dramatic decrease in both short and long chain polyphosphates. Cytologically, the phosphate starvation period was characterized by the development of areas of medium electron density, and vacuolization resulting from expansion of intrathylakoidal spaces.

Inoculation of the phosphate-starved algae into a medium containing a known amount of phosphate led to increases in all phosphorus-containing fractions, particularly the polyphosphates. Increases in both short and long chain polyphosphates were greater than an order of magnitude. The satisfaction of the "phosphorus debt" was met essentially within an hour. Examination of the cells revealed that the cells develop polyphosphate bodies in the characteristic areas of medium electron density that develop during phosphate starvation or phosphate limitation. X-ray energy dispersive analysis of the polyphosphate bodies confirmed that they are deposits consisting of two major elements, phosphorus and calcium.

The alga was not able to utilize the condensed phosphate, sodium tripolyphosphate, without prior hydrolysis of this compound to orthophosphate.

SECTION III

CONCLUSIONS

On the basis of experiments described in the main text of this report, the following conclusions have been derived.

Physiological and cytological aspects of phosphate utilization by the blue-green alga Plectonema boryanum were studied. It was found that the external phosphate concentration influenced the distribution of phosphorus-containing compounds as well as cell architecture.

Culturing the alga in concentrations of 10, 100, and 1000 mg PO4 per liter resulted in increases in the levels of acid-insoluble and acid soluble polyphosphates. The values reported for 100 and 1000 mg PO_4/liter were the same, indicating that the algae were able to assimilate and utilize only fixed amounts of phosphates. This value was calculated to be 6.5 µg P per 10^6 cells. Increased external phosphate concentration led to increases in size and in the frequency of encountering polyphosphate bodies in the cells. However, cell death and lysis also increased at these higher concentrations.

When the algae were grown in a concentration of 1 mg PO_4/liter, the phosphate present in all cell fractions decreased. Cytologically, the cell was also altered. Large areas of medium electron density developed, and the area of intrathylakoidal spaces increased.

The changes that were encountered in the cells grown in 1 mg PO_4/liter were similar but reduced, when compared to algae grown in phosphate-free medium for 5 days. It was determined that cells grown in the absence of phosphate for five days had total cell phosphorus levels of 0.76 µg per 10^6 cells. Cells in culture for two months or longer were found to have total cell phosphorus levels of 0.73 µg P/10^6 cells. This was determined to be the medium cell phosphorus level, limiting growth. Transfer of cells from either of the two culture conditions previously described to a medium containing phosphate led to an "overplus" phenomenon.

The phosphate overcompensation reaction was characterized by increases in all cell phosphorus fractions. The most dramatic increase was that of both soluble and insoluble polyphosphates. These fractions often increased by more than an order of magnitude. The greatest phosphate uptake occurs within one hour of transfer of phosphate starved cells into a medium containing a known amount of phosphate, and is essentially complete at four hours. Phosphate uptake is light dependent and is inhibited by 2,4-dinitrophenol, mercuric chloride, and sodium fluoride. It is suggested that the energy required for the uptake process is the result of the metabolism of photosynthetic products. The total cell phosphorus levels for uptake never increased beyond 18.9 μg P/10^6 cells.

The phosphate overplus phenonemon was also quite distinct cytologically. Large polyphosphate bodies developed in the areas of medium electron density. These areas developed during phosphate-free or phosphate-limited growth. The areas of medium electron density, and consequently, polyphosphate bodies developed in five different locations in the cell: 1. In ribosomal areas, 2. intrathylakoidally, 3. in nucleoplasmic areas, 4. in polyhedral bodies, and 5. in apparent association with strands of DNA. All developmental stages of polyphosphate bodies can be found during this uptake process.

It was also determined that the alga could not utilize sodium tripolyphosphate, a condensed linear phosphate, for the uptake phenomenon. It appeared that hydrolysis of the condensed phosphate was essential for subsequent utilization by the alga.

X-ray energy dispersive analysis of polyphosphate bodies revealed that there are two major components of a polyphosphate body--phosphorus and calcium. Magnesium or other elements are not present in amounts that are greater than background emission. The amounts are below the limit of detectability as a major component. Traces of an element would probably be lost in background emission.

SECTION IV

RECOMMENDATIONS

This investigation was limited to a laboratory study of certain aspects of phosphate metabolism in selected blue-green algae. It was not possible during the tenure of this proposal to evaluate the response of a wide variety of blue-green algae to varying amounts of phosphorus. This aspect should be examined and should include the organisms responsible for "blooms". Further work should be carried out to determine if the overplus phenomenon exists in a wide variety of blue-greens. Work should also be directed toward a determination of what other factors will induce the cells to exhibit the polyphosphate overplus phenomenon.

Now that some basic groundwork has been done it should be imperative to determine if blue-green algae in nature will exhibit the rapid uptake phenomenon and how this relates to "bloom" production. Investigations should also be carried out to determine if blue-greens in nature under "bloom" and "non-bloom" conditions have a store of polyphosphate available. Work should also be carried out on factors affecting phosphorus release from polyphosphate bodies.

From a more basic point of view work should be done to ascertain the exact nature of the polyphosphate and whether the calcium associated with it is essential for its formation and subsequent use. It would also be of basic interest to determine if other ions can replace calcium in the polyphosphate bodies.

We suggest that lines of research, such as outlined above, be pursued in future studies to help us understand the complicated relationship between phosphorus and other factors in the environment on the undesirable growth of blue-green algae.

SECTION V

LITERATURE REVIEW

BIOLOGICAL OCCURRENCE OF POLYPHOSPHATE

Polyphosphate has been a subject of controversy for at least twenty-five years. In 1944, Jeener and Brachet noted a massive accumulation of a basophilic substance within yeast cells after the addition of phosphate to a previously phosphate-starved suspension. Wiame (1947a,b, 1949) and Schmidt *et al* (1946) isolated this substance and identified it as polyphosphate. Thus, granules earlier referred to as "metachromatic" or "volutin" granules were actually discovered to be deposits of inorganic polyphosphates.

Polyphosphates have been reported to occur in a variety of organisms from bacteria and blue-green algae to higher plants and animals (Harold, 1966). The occurrence of polyphosphates seems to be related to two distinct nutritional conditions (Smith *et al*, 1954). The first of these is nutrient imbalance. Exhaustion of an essential nutrient can result in the formation of many types of reserve materials in the cell such as poly B-hydroxybutyric acid, glycogen, polyphosphates, or lipids, which are all osmotically inert (Voelz *et al*, 1966). Pesch (1924) showed that *Corynebacterium diptheriae* volutin production increased when the amount of growth was limited on glucose-blood agar medium due to insufficient blood content. Duguid *et al* (1954) found in *Klebsiella aerogenes* that volutin production was increased on inadequately buffered sugar-containing agar medium. Smith *et al* (1954) obtained evidence that volutin formation in *Aerobacter aerogenes* occurred when cultures were limited by nitrogen or sulfur deficiencies, but not carbon, potassium, or phosphorus. In a similar manner, Spitznagel and Sharp (1959) reported that magnesium deficiency interfered with volutin formation, whereas sulfate deficiency promoted volutin formation in *Mycobacterium bovis*.

The second nutritional condition that can stimulate the formation of polyphosphate is the restoration of a phosphorus supply following phosphate starvation. This phenomenon has been referred to as Phosphate-Uberkompensation" (Liss and Langen, 1962) or "polyphosphate overplus" (Harold, 1964, 1965; Harold and Harold, 1963; Voelz *et al*, 1966). The ability of cells synthetically to accumulate phosphorus was first investigated by Ketchum (1939) in the marine diatom *Nitzschia closterium*. He referred to the deficiency as the phosphorus or phosphate debt, and measured this debt by the amount of phosphate absorbed from the medium by the diatom, or by direct analysis of the cells. The magnitude of the phosphorus debt was directly related to the length of time the cells grew in the light in phosphorus-free medium.

Jeener and Brachet (1944) found that in yeast cells, basophilia associated with volutin granules decreased when the cells were grown on a phosphorus-deficient medium, and increased when the cells were transferred to a medium containing phosphorus. Wiame (1947b) demonstrated abundant volutin synthesis when phosphate-starved *Saccharomyces cerevisiae* was transferred to a phosphate-rich medium. This phenomenon has also been studied in such bacteria as Caulobacter (Grula *et al*, 1954), *Klebsiella aerogenes* (Duguid, 1948), *Aerobacter aerogenes* (Smith *et al*, 1954), and *Myxococcus xanthus* (Voelz *et al*, 1966).

Blum (1966) studied several parameters of phosphate uptake by phosphate-starved *Euglena gracilis*. He reported that many features of this uptake system were similar to those of an active transport system. That is, in *Euglena*, phosphate uptake requires an energy source, can be limited by dinitrophenol, is saturable by substrate, and can be competitively inhibited by a phosphate analog, arsenate. Similar evidence for an active uptake process is presented by Kylin (1966) in *Scenedesmus*, by Simonis and Urbach (1963) in *Ankistrodesmus braunii*, and by Borst-Pauwels and Jager (1969) in *Saccharomyces cerevisiae*.

Two exceptions have been reported regarding "luxury consumption" of phosphate and involvement of an active uptake process. Butt and Lees (1960) have reported that phosphorus deficient cells of *Nitrobacter* assimilate orthophosphate from the medium at a slow rate during nitrite oxidation. Whitton (1967), studying phosphate accumulation by *Nostoc* colonies, found that softer *Nostoc* colonies could accumulate phosphate from the environment by non-active means. This accumulation of phosphate was reduced by pre-treatment with chelating agents.

PARAMETERS AFFECTING ACTIVE UPTAKE OF PHOSPHATE

As has been demonstrated many times, the uptake of phosphorus in "luxury consumption" is a process requiring energy. This uptake can be limited by physical and chemical parameters such as light, pH, temperature, concentration gradient, source of available phosphorus, ion effects (Fogg, 1973), and other factors collectively referred to as a "phosphate-sparing" factor (Shapiro, 1968).

Light stimulation of absorption of 32p labelled phosphate has been reported by Talpassayi (1962) for the blue-green alga *Anabaena cylindrica*. Simonis and Urbach (1963) found that pre-illumination of cultures of the green alga *Ankistrodesmus braunii* stimulated subsequent assimilation of phosphate in the dark. Other reports of dark assimilation of phosphate have been made in *Chlorella* by Kanai, Miyachi, and Miyachi (1963), in *Anacystis nidulans* by Batterton and Van Baalen (1968), in several algae by Stewart and Alexander (1971), and Overbeck (1962) who demonstrated a dark "overplus" phenomenon in *Scenedesmus*. Light and/or dark uptake of phosphate has been found to exist in *Selenastrum capricornutum* (Fitzgerald, 1970) and in *Ankistrodesmus braunii* (Kanai and Simonis, 1968). Harris and Riley (1956) suggested that a dark uptake of phosphate may allow replenishment of phosphorus to phytoplankton which become phosphorus deficient during the day. Fitzgerald (1970) interprets these findings as a means of nutrient absorption by algae in areas of low light intensity. The algae could then rise to the photic zone of a lake where growth could later take place.

Phosphate uptake rates can be affected markedly by such physical parameters as stream current, concentration of other nutrients, and the aquatic environment itself (lake water vs. laboratory conditions). Fogg (1973) discusses the influence of motion on uptake of nutrients by planktonic species. Those organisms which are non-motile achieve changes in concentration gradient by sinking or by motion of currents. Motile organisms are able to change their position and can seek out optimal growth conditions; however, they still may be affected by currents. Schumacher and Whitford (1965) found that in a variety of alga, stream currents as low as 1-4 cm/sec increased the rate of phosphate uptake. The concentration of phosphorus in natural waters is usually low, ranging from about 1-20 µg of phosphorus per liter. These low concentrations do not always repress growth. Atkins (1923, 1925) found that the diatom _Nitzshia closterium_ grew well in cultures until it completely utilized all the available phosphate. The growth of the diatom in natural waters appeared to be seasonal; the phosphate concentrations become higher in the winter, accounting for the summer development of phytoplankton. Kuenzler and Ketchum (1962) showed that _Phaeodactylum tricornutum_ was able to take up phosphorus from solutions containing less than 11 nm phosphate, and suggested that concentration levels of phosphate this low do not depress growth rate. Pilson and Betzer (1973), studying the phosphate flux across a coral reef, found that the phytoplankton take up phosphorus at a nearly constant rate, independent of light quantity, or the magnitude of photosynthetic activity.

The normal low value of phosphate in water has led several authors to discuss the possibility of a "phosphate-sparing" factor (Shapiro, 1968) that is, a substance, quite possibly organic, which allows lake water organisms to respond quite differently to varying phosphate concentration. In 1948, Rodhe demonstrated that the diatom _Asterionella formosa_ would grow well in lake water with concentrations of phosphate as low as 0.002 mg/l. This same diatom, under laboratory growth conditions and in a medium consisting of inorganic salts in distilled water, would only grow when the phosphate concentration was 0.20 mg/l. Mackereth (1953) investigated the active uptake of phosphate by this diatom, and found that luxury consumption of phosphate occurred in lake water supplemented with phosphate. No uptake occurred in phosphate solutions (30 µl) made up in distilled water. Mackereth drew no conclusions as to why the diatoms behaved so differently in lake water and in artificial medium. Shapiro (1968), using a similar system, postulated that in his test organism, _Microcystic aeruginosa,_ the concentration of inorganic anions was probably the most important parameter affecting uptake.

Ion effects and pH can also markedly affect uptake of orthophosphate and distribution into the phosphorus-containing compounds of the cell. Both types of experiments indicate that fixed charges on either the plasmalemma or cell wall can prevent an active ion uptake. Ullrich (1972) found that in synchronized cultures of _Ankistrodesmus braunii_, 32_P - labelling is strongly dependent upon the pH of the culture medium. In alkaline ranges and in the absence of CO_2, organic phosphates and ATP are labelled most strongly, whereas polyphosphate labelling is highest in the acidic range. Using the same organism, Ullrich-Eberius (1973) reported that maximal rates of phosphate uptake occurred between pH 5.5 and 6.5, in agreement with Ullrich. Ullrich-Eberius also found that Na$^+$ enhanced phosphate uptake 8 to 9 times in the light and in the dark. Belsky _et_ _al_ (1970) also reported a specific Na$^+$ requirement for phosphate uptake in the marine fungus _Dermocystidium_ _sp_. Ullrich-Eberius and Simonis (1970) investigated the effect of both sodium and potassium ions on phosphate uptake, by _A_. _braunii_. Again, 0.002M sodium chloride increased phosphate uptake, whereas uptake was constant over a long period of time in the presence of 0.002M KCl. The authors, considering these ion effects coupled with pH dependency, suggested that _Ankistrodesmus_ _braunii_ metabolically transports $H_2PO_4^-$, but not HPO_4^{2-}, and that these effects are exerted at the plasmalemma.

Other ion effects have also been reported in the literature. Polyphosphate formation in _Saccharomyces_ _mellis_ is almost completely inhibited by 0.5 M KCl (Weimberg, 1970). Any phosphate assimilated remains as orthophosphate. However, the potassium effect is reversible, and Weimberg suggests that uptake of orthophosphate and subsequent release of polyphosphate are related to changes in the conformation of the cell membrane, the probable site of action of the high concentration of K$^+$. Baker's yeast, on the other hand, seems to have a specific requirement for potassium ions in the process of phosphate uptake (Schmidt _et_ _al_, 1946). In fact, 0.01M potassium ions have an enhancing effect on the assimilation of orthophosphate.

MICROSCOPY OF POLYPHOSPHATE BODIES

Many organisms possess cellular inclusions which stain metachromatically with certain basic dyes (Rosenberg, 1966). These inclusions have collectively been referred to as metachromatic, volutin, or Babes-Ernst granules (Harold, 1966). The term "volutin" was first used by Meyer (1904) who noticed an accumulation of distinctive granules in _Spirillum_ _volutans_. Wiame (1947a,b; 1949) and Schmidt _et_ _al_ (1946) were the first to identify volutin granules as deposits of inorganic polyphosphates.

Polyphosphate bodies or granules have long been confused with other cytoplasmic inclusions. Two controversies developed based on microscopic studies and histochemical staining. Early electron microscope studies showed that many bacteria contained granules which were highly electron scattering, and had smooth, sharply defined margins, as in _Mycobacteria_ (Lembke and Ruska, 1940; Knaysi _et_ _al_, 1951; Mudd _et_ _al_, 1956), _Corynebacterium_ _diphteriae_ (Morton and Anderson, 1941; Konig and Winkler, 1948; Bringmann, 1950), _Staphylococcus_ _flavocyaneus_ and _Neisseria_ _meningitidis_ (Knaysi and Mudd, 1943). Konig and Winkler (1948) first correlated the identity of these electron-scattering granules with the metachromatically staining volutin granules. This was accomplished by examining the same stained films by light microscopy and electron microscopy. Before Konig and Winkler's correlation was made, the electron-scattering granules were frequently believed to be prokaryotic nuclear bodies. This confusion arose out of light microscopy and histochemical staining, and electron microscopy.

Knaysi and Mudd (1943) and Bringmann (1950) found evidence that the electron-scattering granules contained deoxyribonucleic acid and believed they corresponded to nuclear bodies. Similar cytological evidence was presented by Lindegren (1948) who demonstrated that volutin appeared in previously phosphate-starved yeast cells on the "chromosomes" within three minutes.

From a histochemical point of view, the confusion between volutin granules and "nuclear bodies" is quite understandable. The linear array of negatively charged phosphate groups in polyphosphate bodies, and the phosphate backbone of nucleic acids are similar enough to give confusing results in histochemical staining (Fuhs, 1969). Both polyphosphates and nucleic acids are basophilic and stain metachromatically with basic dyes. Polyphosphates are more strongly basophilic, however, and retain a basic dye at a pH as low as 1.0 whereas nucleic acids destain at pH 3.5 (Fuhs, 1969). Ebel *et al* (1958a) have described a technique for staining polyphosphates based on their ability to form an insoluble lead salt either at pH 3.5, or pH 1.0, depending upon the chain length of the linear polymer. Polyphosphates of chain length eight or greater are stained at either pH 3.5 or 1.0, while those with a chain length of fewer than eight retain a stain only at pH 3.5.

It had also been suggested that the electron-scattering granules in bacteria were mitochondrial equivalents. This was based on evidence by Mudd (1953) and Mudd *et al* (1951a,b; 1956) that the electron-scattering granules stain intravitally with tetrazolium salts and Janus green B. They suggested that these granules are organized centers of oxidative-reductive activity like mitochondria of animal cells and, under certain circumstances, are capable of accumulating volutin. Smith *et al* (1954) suggested that volutin may accumulate in more than one kind of cellular structure or inclusion.

In addition to the references already cited, polyphosphate accumulations as distinct granules have been reported by Rosenberg (1966) in *Tetrahymena pyriformis*, by Widra and Wilburn (1959) in *Aerobacter aerogenes* by Talpasayi (1963) in several blue-green algae, by Jensen (1968, 1969) in *Nostoc pruniforme* and *Plectonema boryanum*, by Stewart and Alexander (1971) in several blue-green algae, by Ebel *et al* (1958b) and Keck and Stitch (1957) in a variety of organisms, and by Voelz *et al* (1966) in *Myxococcus xanthus*.

Association of polyphosphates with other cellular inclusions has been reported by several authors. Weimberg and Orton (1965) concluded that the ortho and polyphosphates of *Saccharomyces mellis* are located in the protoplast and occupy a position which is different from that of phosphomonoesterase. Indge (1968) reported that in another yeast, *Saccharomyces carlsbergensis*, polyphosphate was located in the cell vacuole. Polyphosphates deposition in *Myxococcus xanthus* was found to vary in relation to the growth of the organism by Voelz *et al* (1966). They found three areas of deposition in the cells: 1. Dense granules around polysaccharide inclusions in the cytoplasm when phosphate in a final concentration of 5×10^{-2}M was added to cells grown to log phase in 5×10^{-4}M phosphate; 2. Depositions in the cytoplasm which began as dense strands, subsequently forming tightly wound bodies partially or totally surrounded by nuclear fibers, in cells which were grown in phosphate-free medium and replenished with 5×10^{-3} or 5×10^{-2}M phosphate; and 3. Dense strands scattered throughout the cytoplasm in cells grown for 20 hours without buffer, and replenished with 5×10^{-2}M phosphate.

In describing a developmental sequence of polyphosphate bodies in *P. boryanum*, formed under conditions of excess phosphate and continuous light, Jensen (1969) observed that all cytoplasmic inclusions are excluded from the area of polyphosphate body formation. This evidence is supported by Fuhs (1958) who demonstrated cytochemically that the polyphosphate bodies of another blue-green alga, *Oscillatoria amoena*, contained only polyphosphate.

The most frequently observed images of polyphosphate bodies are those of round granules, quite electron dense, and not limited by a membrane. The most thorough descriptions of polyphosphate bodies and their developmental sequences have been made by Jensen (1968, 1969). These descriptions include such features as sublimation under high electron beam intensity, also reported by Drews and Niklowitz (1957), Stewart and Alexander (1971), Konig and Winkler (1948), and Drews (1960). Polyphosphate bodies, due to their dense nature, often fall out or chip out in sectioning, may compress during sectioning, shrink under the electron beam, or "smear" in the direction of sectioning (Jensen, 1968). The general size range of polyphosphate bodies has been reported to be between 0.1 to 2.0 µm (Stewart and Alexander, 1971; Sicko, 1972). Formation of polyphosphate bodies in *Plectonema boryanum*, under conditions of excess phosphate and continuous light, appears to be in the following sequence (Jensen, 1969): 1. Development of electron-lucent areas in the cytoplasm or at the cross walls; 2. Increase in size of this area to approximately the size of a mature, dense polyphosphate body; 3. Development of a porous area of medium electron density in the electron-lucent area and simultaneous deposition of polyphosphate in the cytoplasm; 4. Penetration of polyphosphate into the porous structure, resulting in the usual image of a dense granule. This work has subsequently been confirmed by Stewart and Alexander (1971).

In addition to the descriptions previously cited, several reports have occurred in which polyphosphate is said to be deposited in morphologically different structures. Munk and Rosenberg (1969) reported that *Tetrahymena pyriformis* deposited polyphosphates in spherical granules which appeared to be surrounded by membranes. "Electron-scattering alveolar bodies" were reported by Fisher (1971) in a lichen phycobiont, *Trebouxia erici*. The appearance of these vaculolar granules varied with fixation. The polyphosphate-containing particles of *Micrococcus lysodeikticus* differed markedly in shape and organization from the volutin granules described in other microorganisms. Friedberg and Avigad (1968) described the electron-dense granules ranging from 40-80 nm in diameter as part of a more complex structure. The polyphosphate bodies appeared to be organized around a granulated center in a rosettelike pattern.

Thus it can be seen from the previous discussion that the morphology and location within the cell of polyphosphate bodies seem to vary in different organisms, and under different growth conditions.

ASPECTS OF PHOSPHATE METABOLISM

There are two distinct classes of polyphosphates found in cells: "acid-soluble", polymers which are readily extracted in cold trichloroacetic acid (TCA), and "acid-insoluble", polymers which are not extracted by cold TCA, but may be extracted by a short exposure to hot TCA (Wiame, 1949; Krishnan *et al*, 1957). Yoshida (1955) first reported the preparation of soluble and insoluble polyphosphates from yeast, and found that polymerization grades of acid-soluble and acid-insoluble were 10 and 50, respectively. Thus, the references in the literature are to acid-soluble forms as being short chain polyphosphates, and acid-insoluble forms as being long chain polymers (Harold, 1966; Terry and Hooper, 1970). Kanai and his associates (1963, 1965) and Miyachi and Tamiya (1961), were able further to subdivide these classes of polyphosphates into four fractions, obtained by a modified Schmidt-Thannhauser method. These fractions, obtained by successive extractions, were classified as follows: 1. poly-Pi "A" - cold 8% TCA; 2. poly-Pi "B" - cold KOH at pH 9.0; 3. poly-Pi "C" - 2NKOH, Reprecipitable by neutralizing the extract; and, 4. poly-Pi "D" - 2NKOH, soluble after neutralizing the extract.

The physiological and metabolic functions of the acid-soluble and acid-insoluble polyphosphates also seem to be distinct, and vary as a function of growth. Wiame (1949) first reported that the acid-insoluble form is metabolically more active in the cell, and that it is rapidly and reversibly transformed to orthophosphate. Katchman and Fetty (1955) substantiated this evidence in *Saccharomyces cerevisiae* by demonstrating that the soluble inorganic polyphosphate fraction maintained a steady-state concentration from generation to generation, while the insoluble fraction appeared only in the later stages of logarithmic growth in a nonsteady-state concentration independent of the concentration of the soluble polyphosphates. Katchman and Van Wazer (1954) postulated that the protein-complexing ability of polyphosphates differing in chain length might determine the different metabolic activities of the polyphosphates in yeast.

The differences in the amounts of the two classes of polyphosphates appear to be a function of growth. Early studies by Smith *et al* (1954) and Wilkinson and Duguid (1960) demonstrated that *Aerobacter aerogenes* contains little or no polyphosphate during exponential growth. A similar system appears to operate in *Corynebacterium xerosis*. Hughes and Muhammed (1962) observed an accumulation of polyphosphate in these bacteria during lag phase after transfer to fresh medium, a decrease during the exponential phase, and an accumulation in the stationary phase. Similar results were also reported by Drews (1968), Mudd *et al* (1958), and Winder and Denney (1957). As a contrast to the above systems, Terry and Hooper (1970) found that short chain polyphosphates were present in constant amounts in *Nitrosomenas europaea* throughout growth and had a negligible turnover rate. Acid-insoluble long chain polyphosphates decreased upon transfer to fresh medium, then increased as growth proceeded and remained fairly constant. Terry and Hooper (1970) suggested that rapid hydrolysis of polyphosphate after transfer to a fresh medium was triggered primarily by the higher pH of the fresh growth medium.

It seems evident that the accumulation or degradation of polyphosphate is a function of cell metabolism. Conditions of polyphosphate accumulation under conditions of nutrient imbalance fall into two distinct patterns - (a) the "polyphosphate overplus" phenomenon as previously described, and (b) cessation of nucleic acid synthesis due to exhaustion of an essential metabolite.

Lindergren (1948) first suggested that volutin, which Wiame (1949) had later identified as metaphosphate, was essential for cell division. Sall *et al* (1956, 1958) reported that in *Corynebacterium diphtheriae* accumulation and disappearance of polyphosphate were physiological events related to cell division, with minimal amounts of polyphosphate present after recurrent periods of cell division. Mudd *et al* (1958) demonstrated that there was a competitive relationship between nucleic acid synthesis and the accumulation of polyphosphate in mycobacterial cells. This relationship was studied using ^{32}P and tracing the exchange from labelled polyphosphate to RNA-P. Earlier work by Schmidt *et al* (1956) had demonstrated that in Baker's yeast, acid-insoluble intracellular polyphosphates were utilized as efficiently as orthophosphate present in nutrient solutions for nucleic acid synthesis. Large accumulations of inorganic polyphosphates did occur in these yeast cells under conditions of complete RNA inhibition. Baker and Schmidt (1964) found that in synchronized cells of *Chlorella pyrenoidosa*, there was a recurrent decrease in the polyphosphate level immediately prior to and during nuclear division. This study also demonstrated that accumulations of total nucleic acid phosphorus and acid-insoluble polyphosphate phosphorus exhibited an inverse relationship to each other. Sauer *et al* (1969) obtained similar results with the slime mold, *Physarum polycephalum*. Inhibition of RNA synthesis in the plasmodium by actinomycin D resulted in a marked stimulation of ^{32}P incorporation into polyphosphate. No such correlation was found after inhibition of either DNA synthesis by 5-fluoredeoxyuridine or protein synthesis by cycloheximide.

Evidence which does not support the hypothesis that polyphosphate-phosphorus may serve as a source of RNA-phosphorus comes from the work of Miyachi and Tamiya (1961). They demonstrated that in *Chlorella ellipsoidea* the phosphorus used in the synthesis of DNA and protein was taken primarily from polyphosphates, while that used in the synthesis of RNA, phospholipid, and other polyphosphates was generally taken from an extracellular phosphorus source. This work is supported by evidence from Kanai *et al* (1965) who found that poly-Pi "A" and "C" function as intermediates transferring phosphate from orthophosphate to DNA and phosphoprotein.

Extensive studies on phosphate metabolism have been made by Harold and his coworkers both on *Aerobacter aerogenes* and *Neurospora crassa*. As has been discussed before, exhaustion of an essential metabolite results in a cessation of nucleic acid synthesis. This same situation was found in *A. aerogenes*. When growth and nucleic acid synthesis were blocked by depriving the organisms of sulfate, assimilation of inorganic phosphate from the growth medium resulted in a slow accumulation of polyphosphate (Harold and Sylvan, 1963). This accumulation could be reversed if growth were allowed to resume. That is, polyphosphate was rapidly degraded, and the phosphate was transferred to the nucleic acid fraction. Again, evidence was accumulated for an inverse relationship between nucleic acid synthesis and polyphosphate accumulation (Harold, 1963, 1965; Harold and Harold, 1965). Using a series of mutants of *A. aerogenes* deficient in some aspect of phosphate metabolism, Harold (1966) has arrived at the following scheme for regulation of polyphosphate synthesis: 1. There is little or no deposition of polyphosphate in normally growing cells. Synthesis of nucleic acids inhibits polyphosphate synthesis and stimulates polyphosphate degradation; 2. If growth and nucleic acid synthesis are inhibited by exhaustion of an essential nutrient, polyphosphate degradation is inhibited. Possibly, the competition for ATP is relieved, and the levels of polyphosphate kinase determine the amount of polyphosphate accumulated; and, 3. The basis of the overplus phenomenon is elevated levels of kinase. Cells subjected to phosphate starvation are derepressed in the snythesis of kinase. Thus, exposure to inorganic phosphate results in rapid polyphosphate synthesis.

The second organism investigated by Harold was Neurospora crassa. In 1948, Houlahan and Mitchell observed an accumulation of polyphosphate in various mutants of Neurospora crassa (Harold, 1966). Harold (1960) found that Neurospora contained high levels of polyphosphate, even during logarithmic growth. Exhaustion of a nutrient in the growth medium resulted in an accumulation of polyphosphate at the expense of RNA. During subsequent starvation, polyphosphate was degraded for RNA synthesis. Harold (1962) also found that ATP was a precursor for the overplus phenomenon, but not an intermediate in the degradation of polyphosphate.

The relationship between nucleic acids and polyphosphates is further complicated by studies which indicate that ribonucleic acid and polyphosphate may exist as a complex in several organisms. This complex was first postulated by MacFarlane (1936). RNA-polyphosphate has been demonstrated by Kulaev and Belozuskii (1958) in Aspergillus niger by Chayen et al (1955) in Torulopsis utilis, by Winder and Denneny (1957) in Mycobacteria, by Ebel et al (1958b, 1962) in yeast by Correll and Tolbert (1962, 1964) and by Correll (1965) in Anabaena and Chlorella, and by Wang and Mancini (1966) in Russel wheat. Correll and Tolbert (1962, 1964) found that in Anabaena, the complex accounted for 25-35% of the total phosphorus, and a major portion of the alga's RNA. However, there was an additional 40-50% of the total phosphorus present as uncomplexed polyphosphate. The polyphosphate-RNA complex in Chlorella was more variable; the relative amounts of the complex varied with respect to the synchronized growth cycle. Wang and Mancini (1966) isolated a RNA-polyphosphate fraction from wheat leaves. Leaves fed with ^{32}P phosphate were found to contain this complex with nearly all of the label in the polyphosphate of the complex.

It can be concluded from the previous discussion that there appears to be a correlation between levels of polyphosphate in cells, and the amount of nucleic acids synthesized. In fact, there might be a direct transfer of phosphate between them, coupled with an energy prerequisite. Evidence such as this has led to the following theories of polyphosphate function.

PHOSPHAGEN HYPOTHESIS VS. PHOSPHATE STORAGE

Phosphagen Hypothesis

Much attention has been paid to the role of polyphosphate as a phosphagen. Ennor and Morrison (1958) have defined phosphagens as "those naturally-occurring phosphorylated compounds which function as stores of phosphate-bond energy from which phosphoryl groups may be transferred to ADP to form ATP as a result of enzymatic catalysis." The essential point is the direct formation of ATP at the expense of the phosphagen. Evidence exists in the literature both for and against this hypothesis.

Kornberg (1956) and Kornberg et al (1956) described in detail an enzyme purified from Escherichia coli which reversibly catalyzed the formation of highly polymerized inorganic polyphosphate from the terminal phosphate of ATP, according to the following equations.

$$1.\quad x\mathrm{ATP} + \begin{bmatrix} (\mathrm{PO}_3)n \\ \text{primer} \end{bmatrix} \longrightarrow x\mathrm{ADP} + \mathrm{PO}_3\ (n+1)$$

$$2.\quad (\mathrm{PO}_3)n \ + \ n\mathrm{ADP} \longrightarrow n\mathrm{ATP}$$

Polyphosphate synthesized chemically or enzymically is utilized quantitatively in the phosphorylation of ADP to ATP. Kornberg (1957) discusses the significance of this reaction and concludes that when cellular levels of ADP are high due to metabolic activity, the ADP could be phosphorylated by polyphosphate. Yoshida (1955) measured the heat of enzymic hydrolysis of insoluble polyphosphate and found it to be 10 Kcal per P-O-P linkage. Winder and Denneny (1955), using cell-free extracts of *Mycobacterium smegmatis*, found that this extract was capable of utilizing metaphosphate for the phosphorylation of glycerol in the presence of ATP. Since their experiments were conducted under anaerobic conditions, they concluded that the metaphosphate must have provided the energy for this phosphorylation. The phototrophic *Chlorobium thiosulphatophilum* appears to synthesize polyphosphate when ATP is formed in excess of other requirements by photophosphorylation (Cole and Hughes, 1965). The authors also postulate that under conditions where ATP utilization is high, net breakdown of polyphosphate could occur. Purification and a study of the properties of the enzyme polyphosphate kinase from *Mycobacterium smegmatis* (Suzuki *et al*, 1972) support these earlier results.

Evidence contradicting the role of polyphosphate as a phosphagen is discussed in detail by Harold (1966). Two lines of research are responsible for this interpretation: 1. Harold (1962) has demonstrated that in intact cells of *Neurospora crassa*, polyphosphate does not break down if energy generation is limited or blocked. Similar evidence is provided by Kaltwasser (1962) for *Hydrogenomonas*. Polyphosphate breakdown can occur in yeast treated with iodoacetate, but the pathway is not through conversion to ATP, (Langen and Liss, 1958; Langen, 1965); and 2. In *Aerobacter aerogenes*, there is direct evidence polyphosphate degradation is hydrolytic, resulting in dissipation of the energy-rich bond (Harold and Harold, 1965). From this evidence, Harold (1966) concludes that the hypothesis of polyphosphate as a phosphagen is not tenable.

Polyphosphate as a Phosphorus Reserve

Much evidence has been presented to support the theory that polyphosphate is a storage form of phosphorus. As has already been discussed, insoluble polyphosphate can serve as a source of phosphorus in nucleic acid synthesis, most often RNA synthesis. Polyphosphate, due to its structure, minimizes disturbance of osmotic equilibrium in the cell, and thus makes it an attractive compound for storage in the cell (Voelz *et al*, 1966; Harold, 1966). As will be discussed in a later section, the concentration of phosphorus in the environment is usually low. If the scheme proposed by Harold as discussed previously were operative, cells subjected to low concentrations of phosphorus would have elevated levels of polyphosphate kinase, the enzyme responsible for polymerization of orthophosphate. Thus, when the organisms come into contact with a phosphate source, the organism would be able to accumulate a phosphorus reserve for later synthesis of cellular compounds (Harold, 1966). This theory is also substantiated by Kaltwasser (1962) who found similar results in *Hydrogenomonas*.

ECOLOGICAL ASPECTS OF PHOSPHORUS IN NATURAL WATERS

The role of phosphorus in the eutrophication processes has long been a controversial subject. Nutrients such as carbon, nitrogen, and phosphorus are often referred to as "limiting" or regulating nutrients in an aquatic ecosystem, limiting in the sense that they control photosynthetic productivity. In fact, nutrient concentrations of these and other elements are often used to classify lakes into either oligotrophic or eutrophic categories (Rodhe, 1969).

Eutrophication, in the most popular definition, is usually mancentered, and refers to an enrichment process which is vastly accelerated by man (Hasler, 1947; Likens, 1972). From an ecosystem point of view, eutrophication may be broadly defined as follows (Likens, 1972): "Eutrophication ... nutrient or organic matter enrichment, or both, that results in high biological productivity, and a decreased volume within an ecosystem." Likens (1972) also points out that eutrophication is often considered as a form of pollution, but the two terms are actually not synonymous.

Phosphorus, especially in the form supplied by effluents containing synthetic detergents, is usually implicated as the main source of nutrient enrichment responsible for accelerating eutrophication. This topic, as will be reviewed, is quite extensive, and yet the role of phosphorus, as it involves organisms in an aquatic ecosystem, is not clearly defined.

NUTRIENT SOURCES IN AQUATIC ECOSYSTEMS

Phosphorus can be found in a variety of forms in water. The sources of phosphorus-containing compounds are equally diverse (Table 1). It has been estimated that millions of pounds of phosphates per year are contributed from these sources (Ferguson, 1968). The phosphate source most frequently cited as the principle factor causing blooms of algae is synthetic detergent builders, consisting for the most part, of sodium tripolyphosphate.

Evidence exists again, for both sides of this question. An important factor lies in the definition of a "limiting" nutrient, and changes in the concentration of this nutrient. Gibson (1971) reviews the concept of nutrient limitation from the following three definitions: 1. An organism is limited when it is not growing as fast as it is theoretically able to; 2. A factor is said to be limiting when it is in such short supply that no growth is possible; and 3. A factor is not limiting, if when it is increased, no effect on growth is observed. The third definition is most commonly encountered, and evidence both for and against phosphate being a limiting nutrient is approached from this viewpoint.

Phosphorus has long been found to be an important minimum factor for plant growth in natural waters (Sawyer, 1947, 1952; Ohle, 1953; Fuhs *et al*, 1972). Thomas (1953) observed that phosphorus and nitrogen were minimum factors for algal growth. The high phosphorus content of waste waters was a

TABLE 1. SOURCES OF NUTRIENTS OCCURRING IN NATURAL WATER (Modified from P. J. Weaver, 1969; L. I. Keup, 1968)

	SOURCE	TYPE OF PHOSPHORUS	AMOUNT	REFERENCES
I	Municipal Treatment Plants and Private Waste Disposal Systems	total soluble P output for all types of sewage	1-13 mg/1	Fitzgerald and Rohlich (1953) Lewin (1973)
	A. Domestic			
	1. Human Wastes	Ortho P-P	10 mg/1	Mitchell (1971)
	2. Soaps and Detergents	Organic	total estimated all sources	
	3. Household food	(2) condensed inorg. PO_4	3 lb. mean per capita per annum	Bush and Mulford (1954) Devey and Harkness (1973)
	B. Industrial			
II	Industrial Wastes Discharged directly to Waterways			Solt (1973)
III	Water Treatment Chemicals			
IV	Land Runoff			
	A. Urban Runoff and Drainage			
	B. Stormwater	measured as P	0.82 lbs. PO_4-P/yr/acre, 10-1400 µg/1	Weibel *et al*, (1966) Sylvester (1961)
	C. Rural Runoff and Drainage			
	1. Agricultural			
	a. soils (erosion)	measured as P	13 lbs./acre	Fippen (1945)

b. fertilizers	slurries containing P		Cooke and Williams (1973)
c. animal excrement		up to 1.5mg/l	Cooke and Williams (1973)
2. Non-agricultural land			
a. decaying leaves	orthophosphate	54-230µgP/ gram leaves	Cowan and Lee (1971)
b. wild animal wastes			
V Groundwater	PO_4-P	10-70µg/l	
VI Reserves in Lakes			
A. Bottom Muds	Organic, PO_4-P		Golterman (1973)
B. Living Aquatic Organisms			
C. Lake Water			
D. Peat	PO_4-P	up to 1mg/l in peat-rich water	Golterman (1973)
VII Atmosphere			
A. Rainfall	not determined	0-49µg/l 0-30µg/l	Hutchinson (1957) Wiebel, *et al* (1966)
B. Dustfall			

key factor in the acceleration of eutrophication above its natural rate. If the phosphorus demand of the algae had been met, nitrogen replaced phosphorus as the minimum factor. Fuhs et al (1972) also observed multiple nutrient limitation in Lake George, New York. They found that nitrogen and phosphorus could act as limiting nutrients, either simultaneously or alternating with time and space. With nitrogen limitation in effect, low concentrations of phosphorus could not be interpreted as indicating phosphorus limitation, because nitrogen limitation favored luxury uptake of phosphorus. Schelske and Stoermer (1972) found that in natural phytoplankton assemblages enclosed in plastic bags in Lake Michigan, phosphorus was the limiting nutrient. This resulted in lower concentrations of silica in the lake, and replacement of diatoms with nonsiliceous forms, such as blue-green and green algae. Edmunson (1961, 1969, 1970, 1972) demonstrated that the phytoplankton population of Lake Washington (Seattle) was dependent upon the increasing volumes of effluent from secondary sewage treatment plants. There was a strong correlation between the abundance of phytoplankton in the summer, and high levels of phosphate, but not nitrate or carbon dioxide, in the water during the winter months. Diversion of the effluents resulted in a decreased phosphate concentration, and a reversal of cultural eutrophication in Lake Washington. In fact, by the time half the effluent had been diverted, the phosphate concentration started to decrease. Edmunson (1972) then made predictions about the possible changes that could occur. Since about one-half of the phosphate in the sewage came from detergents, elimination of all detergents from sewage should lead to the same results as following diversion of one-half of the effluent. The lake could then tolerate a much larger human population without their accompanying detergents. Similar results in determining in situ effects of added phosphates in natural waters were obtained by Powers et al (1972). Lakes of varying productivity in both Minnesota and Oregon were studied, and it was found that phosphorus appeared to be the primary controlling nutrient in enrichment experiments. They postulated that several lakes could be restored by the removal of phosphorus from municipal waste by advanced treatment methods. Sonzogni and Lee (1972) also demonstrated that effluent diverted from a number of Wisconsin lakes resulted in a reduction of phosphorus content of the lakes, as well as a decrease in the frequency and severity of blue-green algal blooms. Pitcairn and Hawkes (1973) demonstrated that there was a general positive correlation between the standing crop of Cladophora and the phosphorus concentration of several river waters. They confirmed the importance of phosphorus by showing that the growth of Cladophora in waters upstream of sewage discharges could be increased to downstream levels by the addition of phosphorus.

Taylor (1967) reviewed the phosphorus concentrations that limit algal growth. Sawyer (1952) found that phosphorus concentrations below 0.01 parts per million severely limit algal growth, while concentrations of 0.05 parts per million or higher permit profuse growth. Most uncontaminated lakes contain between 0.01 and 0.03 parts per million of phosphorus; thus, adding a relatively small amount of phosphorus to the lakes, to increase the level to 0.05 ppm, is likely dramatically to increase productivity (Sawyer, 1952).

This view is not supported by a number of workers. Kuentzel (1969) reviewed the carbon dioxide-phosphate controversy and concluded that CO_2 and organic matter supporting growth of bacteria were responsible for massive algal blooms rather than phosphorus. He suggested that a symbiotic relationship between bacteria and algae could open up another possibility for reductions in algal growth via control of bacteria. The line of succession suggested was: organic matter ⟶ bacteria ⟶ CO_2 ⟶ algae. This review was attacked heavily by Shapiro (1970) who pointed out the fact that CO_2 is not limiting, and in spite of high concentrations of CO_2, algae are not able to grow unless there is a sufficient supply of phosphorus.

Mitchell (1971) found that the eutrophication potentials of a phosphate-containing detergent and two phosphate-free detergents were not significantly different. He pointed out that elimination of phosphates in domestic sewage due to detergents would still result in a concentration of 3-4mg P/l, using conventional primary water treatment. He did not consider the use of activated sludges. His estimates of phosphate concentrations in domestic sewage are in agreement with those of Hudson and Marson (1970), and Marson (1971). However, Marson reported up to 90% phosphorus removal by any of the precipitation processes currently available, and 80% by luxury consumption by activated sludge.

Levin and Shapiro (1965) demonstrated that luxury uptake of orthophosphate by sludge organisms occurred in the absence of growth. Uptake was dependent upon the dissolved oxygen and pH of the mixed liquor, with maximum uptake occurring in the pH range 7.0-8.0. Shapiro (1967) later determined that settling of activated sludge in settling ponds of water treatment plants led to anoxia of the organisms, and subsequent release of the phosphate. Yall et al (1970) supported the concept of biological luxury uptake by activated sludges using both radioactive tracers and inhibitor studies.

Jenkins et al (1973) studied the environmental impact of detergent builders in California waters, and found that detergents accounted for about 35% of the total phosphorus released to surface waters. They suggested that the algae in the California waters did not appear to be phosphorus-limited, so that control of phosphorus input would do little to control eutrophication. If phosphate control were necessary, they suggested point-source elimination for a particular area and not solely detergent phosphate elimination.

The controversy of phosphate-removal still exists. The problem is further complicated by the fact that snythetic detergent builders are condensed phosphates, usually sodium tripolyphosphate (Davis and Wilcomb, 1967, 1968). The studies discussed so far were concerned with utilization of orthophosphate in natural waters by phytoplankton. Thus, two more problems are involved: 1. The natural hydrolysis of condensed phosphates in natural waters, and 2. Uptake and metabolism of condensed phosphates by phytoplankton.

HYDROLYSIS OF CONDENSED PHOSPHATES

Synthetic detergent builders, condensed phosphates, appear in sewage effluents and receiving waters. These compounds, under various conditions, have a tendency to react with water and ultimately form orthophosphate. Engelbrecht and Morgan (1959) found that sodium tripolyphosphate, and tetrasodium polyphosphate were subject to degradation in natural waters, the hydrolysis rates varying with the water sample. They also indicated that biological life exerted an effect on the rate of degradation, since filtration of raw water decreased the rate of degradation.

Clesceri and Lee (1965a,b) studied the rates of hydrolysis of condensed phosphates in non-sterile and sterile environments. They found that pyrophosphate and tripolyphosphate were apparently not as available a phosphorus source as orthophosphate. Using both unialgal and axenic cultures of Chlorella, the condensed phosphate compounds were completely hydrolyzed in a short time by the unialgal cultures, but not by the axenic cultures. They attributed the increased hydrolysis rates to the presence of non-algal microorganisms in the unialgal culture. Clesceri and Lee (1965b) also showed that the rate of hydrolysis of condensed phosphates was higher by several orders of magnitude in sterile lake water and algal culture media than in distilled water at a similar pH and temperature. Hydrolysis rates of both pyrophosphate and tripolyphosphate were highest in those solutions containing the highest concentrations of calcium ion. When comparing these rates to those determined in the presence of microorganisms under similar conditions (Clesceri and Lee, 1965a) it was found that enzymatic processes controlled the aqueous environmental chemistry of the condensed phosphates.

Clesceri and Lee (1965a) summarized the studies on the factors that influence the rate of condensed phosphate hydrolysis as follows:

	Factor	Effect on rate
A.	Temperature	10^5-10^6 faster from freezing to boiling.
B.	pH	Rates of hydrolysis of all condensed phosphates are higher in acidic media; tripolyphosphate hydrolysis can be base-catalyzed, but it is most stable in the pH 9-10 range.
C.	Enzymes	Divalent cations in combination with enzymes accelerate hydrolysis, especially magnesium; adaptive ènzymes, usually phosphatases, are produced by many organisms.
D.	Colloidal gels	Hydrated oxides of iron, cobalt, nickel, aluminum, and rare earths accelerate hydrolysis.
E.	Complexing cations	pH effect is absent when there are no cations; calcium increases hydrolysis rates more than sodium.
F.	Concentration	Hydrolysis of condensed phosphates are first order processes; the rates are proportional to the concentration.
G.	Independence of hydrolysis in a mixture of phosphates	Hydrolysis of different species in the same dilute solution proceeds independently.

The data presented by Clesceri and Lee (1965a,b) were later substantiated by Davis and Wilcomb (1967, 1968). Cultures of several green algae, grown in nutrient medium with phosphate concentrations close to those expected in sewage, were capable of degrading polyphosphate to orthophosphate. The rate of orthophosphate assimilation was dependent upon the environment. Axenic cultures of the algae demonstrated hydrolytic ability, a greater hydrolytic capacity existing in moving systems than in static ones. Davis and Wilcomb (1968) also demonstrated that several genera and species of blue-green algae were able to utilize condensed phosphates, and, in fact, return them to the aqueous environment during certain growth phases. Again, uptake of the condensed phosphates by the blue-greens was greater in moving columns than in static cultures.

It has already been suggested (Clesceri and Lee, 1965a) that microbial activity is responsible for the degradation of condensed phosphates to a much greater extent than other environmental parameters. This microbial activity appears to be the result of phosphatases, enzymes which catalyze the release of bound phosphates. Hydrolysis of tripolyphosphate results in a unit of pyrophosphate and a unit of orthophosphate, whereas hydrolysis of pyrophosphate yields two units of orthophosphate (Clesceri and Lee, 1965a). Kornberg (1956) isolated tripolyphosphatase, pyrophosphatase, and trimetaphosphatase from yeast cells. Eppley (1962) found that orthophosphate was released when living pieces of _Porphyra_ were incubated with several condensed phosphates and ATP. Eppley (1962) indicated enzymatic catalysis of these compounds, and also concluded that both calcium and magnesium were essential for hydrolysis.

The presence of adaptive phosphatases has been observed in a variety of organisms. Galloway and Krauss (1963) found an adaptive pyrophosphatase associated with the cell wall of _Chlorella_. Overbeck (1961a,b) found intercellular phosphatase activity in _Scendesmus quadricauda_ when the substrates were condensed or organically bound phosphates. A number of correlations have been made numerous times and in a variety of organisms between the levels of alkaline phosphatase and the amount of phosphate in the growth medium. Alkaline phosphatase appears to be induced when the external phosphate source is limiting in _E. coli_ (Torriani, 1960), _B. subtilis_ (Cashel and Freese, 1964), _Vibrio parahaemolyticus_ (Sakaguchi _et al_, 1972), _Anacystis nidulans_ (Reichardt, 1971), and _Anabaena flos-aquae_ (Bone, 1971), and a variety of algae, (Fitzgerald and Nelson, 1966). Torriani (1960) suggested that it is a means of obtaining phosphate from organic phosphate when the supply or concentration of orthophosphate becomes limiting in the medium.

SECTION VI

MATERIALS AND METHODS

GROWTH CURVES

The test organism was chosen after series of growth curves in various culture media were determined. The two blue-greens initially selected were _Plectonema boryanum_ Gomont (Indiana Culture Collection No. 581) and _Oscillatoria tenuis_ Ag. (Indiana Culture Collection No. 428), obtained from the Starr Culture Collection (Starr, 1964). This selection was based on their ability to grow well in a defined medium, Modified Fitzgerald (Fitzgerald _et al_, 1952; Zehnder and Gorham, 1960). Growth curves for the algae were determined by inoculating a known dry weight of the algae into sterile culture tubes containing 20 ml of Modified Fitzgerald's medium. The tubes were then placed in a Sherer-Gillette growth chamber adjusted to: 1. 500 ft-candles of illumination (5330 lux) from incandescent and fluorescent sources; 2. 25°C; and, 3. an alternating 12 hour day/night cycle. Growth was monitored for a period of 28 days as a function of increase in dry weight per unit volume. Stock cultures were also maintained under these conditions for one month, and subsequently placed under 200 ft-candles of illumination. Transfers to fresh medium were made from cultures which were between two and four months old.

DRY WEIGHT DETERMINATIONS

Dry weight of the samples was found to be the most consistent and easiest method for determining the growth of the algae. Both blue-greens tested are filamentous and have sheaths which make dispersal and rupture of filaments into individual cells difficult for counting or spectrophotometric analysis. Millipore filters, type HA with a pore diameter of 0.45 μm predried by heating to 100°C for 24 hours, and weighed after cooling to determine the dry weight. Known volumes of algal suspensions were then syringed sequentially ten times each through 14, 18 and 22 gauge sterile disposable needles, and then passed through the predried Millipore filters. After drying the filter under an incandescent lamp, the filters were then dried again under the previously described conditions, and weighed when cool. The difference in the weight of the filters was taken to be the dry weight of the algae per unit volume filtered. Normally a 10 to 20 ml aliquot containing between 200 and 1000 mg algae per liter was filtered.

CORRELATION OF DRY WEIGHT TO CELL COUNTS

Although dry weight analysis was chosen to be the method for quantitating the algal suspensions, correlations were made between actual cell number and dry weight. This was accomplished by extensive syringing of the cultures through 22 gauge needles and recording the following information using a hemocytometer and reticule: 1. number of algal filaments per unit volume of culture; 2. length of individual filaments; and, 3. number of cells per filament. In this manner, the number of cells per unit volume was determined, and correlated to a dry weight analysis of that culture. Analysis of cell number, dry weight, and average cell size were made for logarithmic phase cultures, phosphate starved cultures, and cultures at the end of the four hour rapid uptake period.

PHOSPHATE ASSAY AND CALIBRATION

Phosphate depletion of the medium was also monitored during all growth determinations. This was accomplished by testing the growth medium without the algae. In all cases, the algae were removed by Millipore filtration as previously described. A known volume of the filtrate was diluted volumetrically so that the spectrophotometric absorbance values were in the range 0.3 to 0.7 whenever possible. The two tests used for the determination of orthophosphate were the stannous chloride method (American Publ. Health Assoc. 1965) and the single solution method of Murphy and Riley (1962).

The method selected as the most reliable was that of Murphy and Riley (1962). The orthophosphate determinations are colorimetric tests; the absorbance of the phosphomolybdate complex formed is a function of orthophosphate concentration in solution. The colored complex formed during the Murphy-Riley determination is stable for a period of 24 hours, and there is less interference due to arsenic or salts. The stability of the colored complex formed in the stannous-chloride method is considerably less. Forty ml of the solution to be tested were placed in a 50 ml graduated cylinder. Eight ml of the mixed reagent, and two ml of glass distilled water were added so that the final volume of the solution was fifty ml. The solution was then mixed thoroughly, and color development was allowed to proceed from at least 10 minutes up to a period of 1 hour. Percent transmittance was read directly and converted to an absorbance value. All readings were taken at 880 nm on a Spectronic 20 spectrophotometer equipped with infrared sensitive phototubes and filters. Calibration curves were used to determine the sensitivity of the test. The absorbance value divided by the slope of the calibration curve gave a direct reading of phosphorus concentration as phosphate. All phosphate determinations were carried out in glassware which was acid washed and used only for these tests.

Calibration curves were determined in the following manner: A standard phosphate solution containing 0.1757 grams of potassium dihydrogen phosphate per liter was prepared. This solution contains 40 mg P (as phosphate) per liter. Dilutions of the stock solution were made to the following concentrations:

80 µg P/l (3.2 µg P/40 ml)
160 µg P/l (6.4 µg P/40 ml)
320 µg P/l (12.8 µg P/40 ml)
400 µg P/l (16.0 µg P/40 ml)
640 µg P/l (25.6 µg P/40 ml)

The percent transmittance values were determined, and converted to absorbance.

The values were measured at 880 nm in 2.56 cm cells. Beer's law is obeyed in this concentration range, and the method gives an excellent reproducibility.

STARVATION CONDITIONS

Cells of Plectonema boryanum were grown in Modified Fitzgerald's medium containing 8 - 10 mg PO_4 per liter for 14-17 days at 25°C, 500 ft-candles of illumination, and an alternating 12 hour day/night cycle. To induce phosphorus starvation, cultures were harvested aseptically by centrifugation in a Sorvall refrigerated centrifuge at 21°C and 12,100 xg for 10 minutes. The medium containing phosphate was decanted, and the cells were washed 3 times in sterile medium free of phosphate. After the final centrifugation, the cells were resuspended in phosphate free medium and then placed under the original environmental conditions. Optimal starvation conditions were determined by prolonging starvation up to a length of 12 days.

"OVERPLUS" OR RAPID UPTAKE

Luxury storage of phosphate was induced by starving the algae of phosphate for 5 days, and then inoculating into medium containing a known amount of phosphate. This phosphate concentration was varied in the range 0.1 to 112 mg PO_4 per liter. The rapid uptake was monitored in one or more of several ways: 1. increase in dry weight of the algae; 2. depletion of phosphate from the medium; 3. increase in total phosphate in the cells; 4. increase in various phosphate-containing fractions; or, 5. light or electron microscopy of the samples.

INHIBITORS AND TEMPERATURE STUDIES

For uptake studies involving inhibitors or temperatures other than 25°C, the algae were treated in the following manner. At the end of the starvation period, the algae were pelleted by centrifugation and resuspended in medium containing no phosphate and containing the appropriate concentration of inhibitor. The algae were then placed under the appropriate growth conditions and incubated with the inhibitor for a period of 1 hour prior to the addition of phosphate in the normal uptake manner. The three inhibitors tested were 2,4-dinitrophenol (5×10^{-3}M), mercuric chloride (1×10^{-2}M), and sodium fluoride (1×10^{-3}M). Uptake studies involving two different temperatures were treated somewhat differently, depending on the temperature. For uptake at 37°C, the algae were first acclimated to that temperature for a period of one month, then logarithmic phase cells were starved in the normal manner. This was possible since the algae grow well at this temperature. Uptake at low temperatures involved the growth of the algae at 25°C, and preincubation of the algae at 4°C for 1 hour prior to uptake. This procedure was followed because there is virtually no growth at this low temperature.

TOTAL PHOSPHATE DETERMINATIONS

Batterton and Van Baalen's (1968) modification of the Menzil-Corwin (1965) potassium persulfate ($K_2S_2O_8$) digestion was used for the assay of total phosphorus levels in the cells. A known aliquot of washed cell suspension containing no more than 1000 mg algae per liter was placed in screw cap culture tubes containing 0.5 grams of potassium persulfate. The tubes were then sealed loosely and autoclaved at 121°C for thirty minutes. The clear contents of the tube were transferred quantitatively and assayed for orthophosphate by the Murphy-Riley technique. Total phosphorus was reported as µg P per µg dry weight algae.

EXTRACTION SCHEME FOR POLYPHOSPHATES

The extraction scheme chosen was Harold's (1960; 1963) modification of the Schmidt-Thannhauser extraction scheme for nucleic acids (1945). Both trichloroacetic acid (TCA) and perchloric acid were tested initially. TCA was found to be more reproducible, and was chosen subsequently for all extractions. A flow chart of this scheme is presented in Figure 1.

Cold TCA Extraction

Washed algal suspensions were extracted twice with 5 ml of cold (4°C) 5% TCA. The supernates were pooled along with the distilled water wash of the residue. This supernate was assayed both for orthophosphate and total phosphate. In all cases, the residue was separated from the supernate by a centrifugation in a VWR MSE GT 2 centrifuge with a swinging bucket rotor at 3280 rpm (1745 xg) for 10 minutes.

Lipid Extraction

The residue of the cold TCA extraction was then extracted with 5 ml of absolute ethanol at room temperature for 30 minutes, followed by extraction in 5 ml of ethanol-ethyl ether (3:1). The mixture was boiled for 1 minute, and left to stand at room temperature for 20 minutes. The two supernates were pooled along with a distilled water rinse of the residue and assayed both for orthophosphate and total phosphate.

Hot TCA Extraction

The lipid-free residue was then dried by heating the test tubes in a 70°C water bath, and then extracted twice with 5 ml portions of 5% TCA warmed to 70°C at 15 minute intervals. The supernates were pooled along with a distilled water rinse of the residue and assayed both for orthophosphate and total phosphate. The residue remaining after this extraction was then digested with potassium persulfate as previously described, and then analyzed for orthophosphate content.

Separation of Nucleic Acids

200 mg of sulfuric acid washed Norit A was added per 5 ml of extract. Nucleic acids were then eluted with alcoholic ammonia from the charcoal, and either analyzed for total phosphorus concentration, or the extinction at 260 nm was determined.

For all total extractions, the total phosphate content of the algal suspension was determined before equivalent aliquots were fractionated. In this manner, percent recovery could be calculated. Secondary precautions were also taken by total phosphate determinations on residues after each extraction, and

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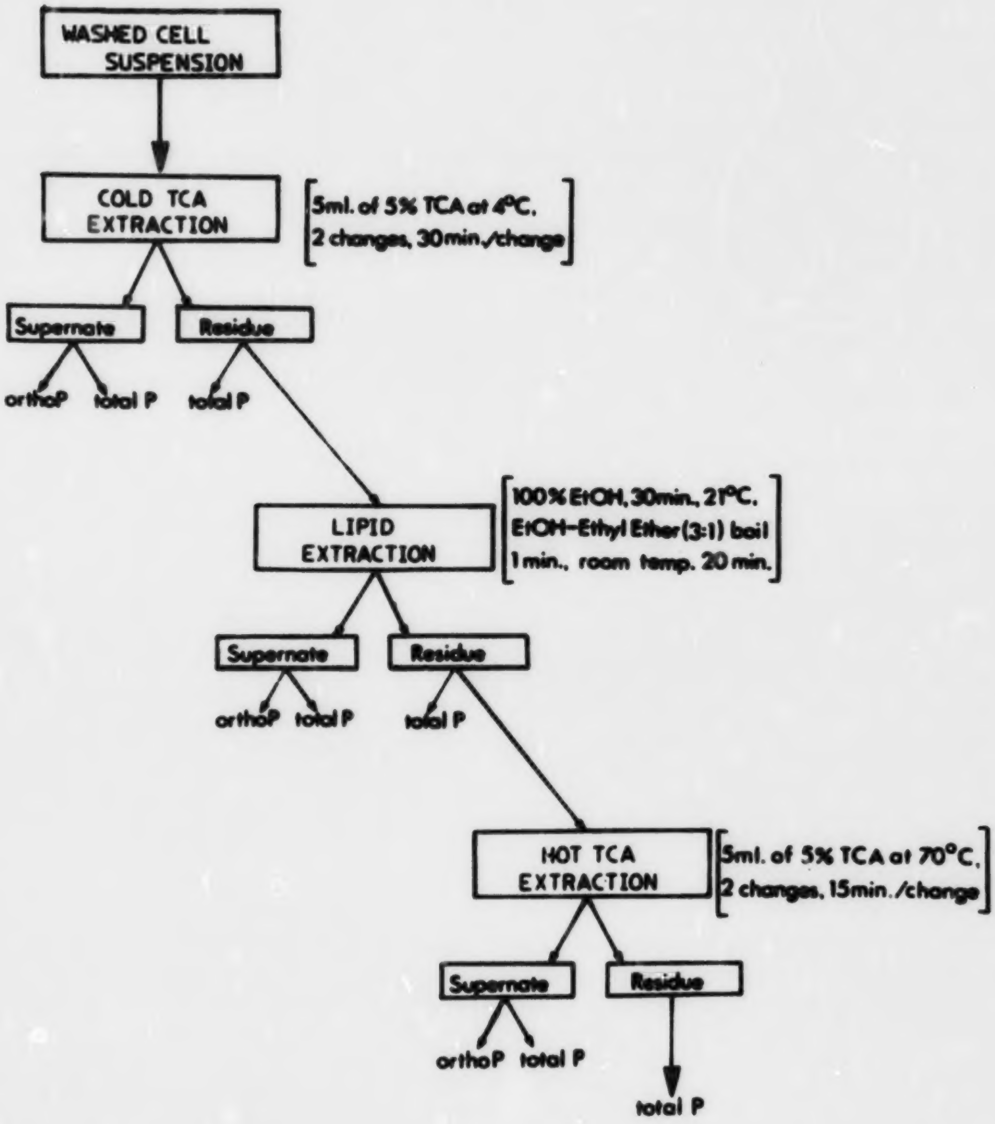

WASHED CELL SUSPENSION
COLD TCA EXTRACTION
5ml. of 5% TCA at 4°C, 2 changes, 30min./change
Supernate
Residue
orthoP
total P
total P
LIPID EXTRACTION
100% EtOH, 30min., 21°C, EtOH-Ethyl Ether (3:1) boil 1min., room temp. 20 min.
Supernate
Residue
orthoP
total P
total P
HOT TCA EXTRACTION
5ml. of 5% TCA at 70°C, 2 changes, 15min./change
Supernate
Residue
orthoP
total P
total P

Figure 1. Flow chart of the extraction scheme for polyphosphates.

by processing triplicate samples in all cases.

ELECTRON MICROSCOPY

Algae were harvested from cultures grown under the various culture conditions previously described, and prepared for electron microscopy in the following manner. Algae were pelleted from the original medium by centrifugation at 1745 xg. The supernatant medium was discarded and the algae resuspended in Modified Kellenberger according to the method of Pankratz and Bowen (1963). The fixative employed was 1% OsO_4 in Michaelis buffer of pH 6.2 for 3 hours at room temperature. The algae were then dehydrated in a graded ethanol series and embedded in Epon 812 according to the method of Luft (1961).

Sections approximately 500 A thick were cut with a DuPont diamond knife on a LKB Ultrotome III, and collected on clean 300 mesh copper grids. The sections were post stained with saturated uranyl acetate in methanol (Stempak and Ward, 1964) for 10 minutes or with lead salts (Reynolds, 1963), separately or in combination with uranyl salts, and examined in an Hitachi HU 11E electron microscope operating at 75 kv. Pictures were taken on Kodak Contrast Projection Plates and developed with D-19.

LIGHT MICROSCOPY

Algal suspensions were stained for polyphosphates by the method of Ebel *et al* (1958a) and Jensen (1968). This staining procedure employs the ability of lead salts to complex with polyphosphate and remain stable at low pH values. The algae were initially fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer adjusted to pH 6.2 for a period of 1 hour at 4°C. The algae were then rinsed five times in 0.1 M cacodylate buffer, pH 6.2, and incubated in 20% lead nitrate, adjusted to pH 3.4 with acetic acid, for 4 hours at room temperature. After the incubation in lead nitrate, the algae were washed thoroughly five times with distilled water, then placed in 1% ammonium sulfide at room temperature for ½ hour. They were again washed with distilled water, and then examined with a Zeiss light microscope (Jensen, 1968). Pictures were taken either with Polaroid 55 PN film, or Tri-X film and developed in diluted Microdol X.

X-RAY ENERGY DISPERSIVE ANALYSIS

Sections, approximately 0.5 μm in thickness, were cut on glass knives and mounted on 200 mesh copper grids. The Sections were then examined and analyzed in a JEM-100B analytical electron microscope fitted with a scanning attachment, a ± 60° side entry goniometer stage, and either EDAX or KEVEX energy dispersive X-ray analysis systems having 180 eV resolution. For orientation purposes, the sections were examined at either 80 or 100 KV in the transmission or scanning modes. The areas to be analyzed were then selected, and the accelerating voltage reduced to 40 KV for elemental analysis. The beam spot can be reduced to less than 50A in diameter, so the beam was in all cases reduced to the size of the polyphosphate body, or a comparable area if no polyphosphate body was present.

All analyses were carried out in one of 2 ways: either total counts of emitted X-rays were read for a fixed time period, or total counts, an arbitrary number, and regardless of time, were read. The fixed time was usually 100 seconds, and the fixed count was usually 20,000.

Samples examined were treated in three ways: 1. Glutaraldehyde - fixation and Epon embedding; 2. Osmium - fixation and Epon embedding; and, 3. Osmium - fixation and Durcupan embedding. Durcupan is a water-soluble embedding medium. All samples examined were starved of phosphate for five days, and fixed after four hours of rapid uptake.

SECTION VII

RESULTS

GROWTH CURVES

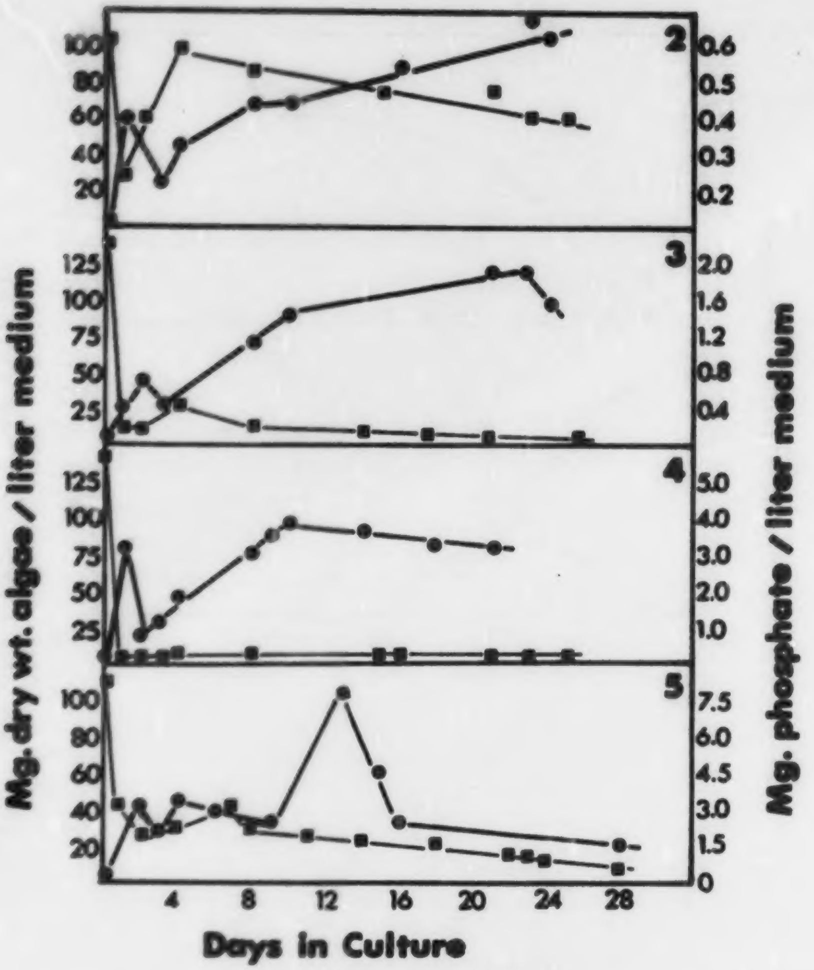
The test organism, *Plectonema boryanum,* was chosen after a series growth curves were determined both for *P. boryanum* and *Oscillatoria tenuis*. *P. boryanum* is able to grow well with phosphate concentrations ranging from 0.8 mg PO_4 to 1100 mg PO_4 per liter (Figures 2-8). The growth of the alga was also monitored simultaneously for phosphate depletion of the medium by the alga. In all phosphate concentrations monitored, the phosphate remaining in the medium was reduced substantially the first day after transfer, rose slightly on the third or fourth day, and then decreased gradually through the remainder of the 28 day period (refer to figures 2-8).

The growth curves varied somewhat with the different concentrations. All growth curves were characterized by an increase in dry weight, often more than four-fold, on the first day after transfer. This initial peak then diminished during the lag period. The growth of *Plectonema boryanum* is sigmoidal; lag, log, stationary, and decline phases are recognizable. The length of the lag and log periods seem to be a function of the phosphate concentration in the medium. Longer log periods of growth are encountered at higher phosphate concentrations (Figures 6 and 7). Lag period appears to be longer at lower phosphate concentrations.

This data led to routine culture of *P. boryanum* at either 8 or 10 mg of phosphate per liter. At these concentrations, log phase occurs approximately between 13 and 17 days of culture (Figures 5 and 6). These are also normal phosphate levels for synthetic media.

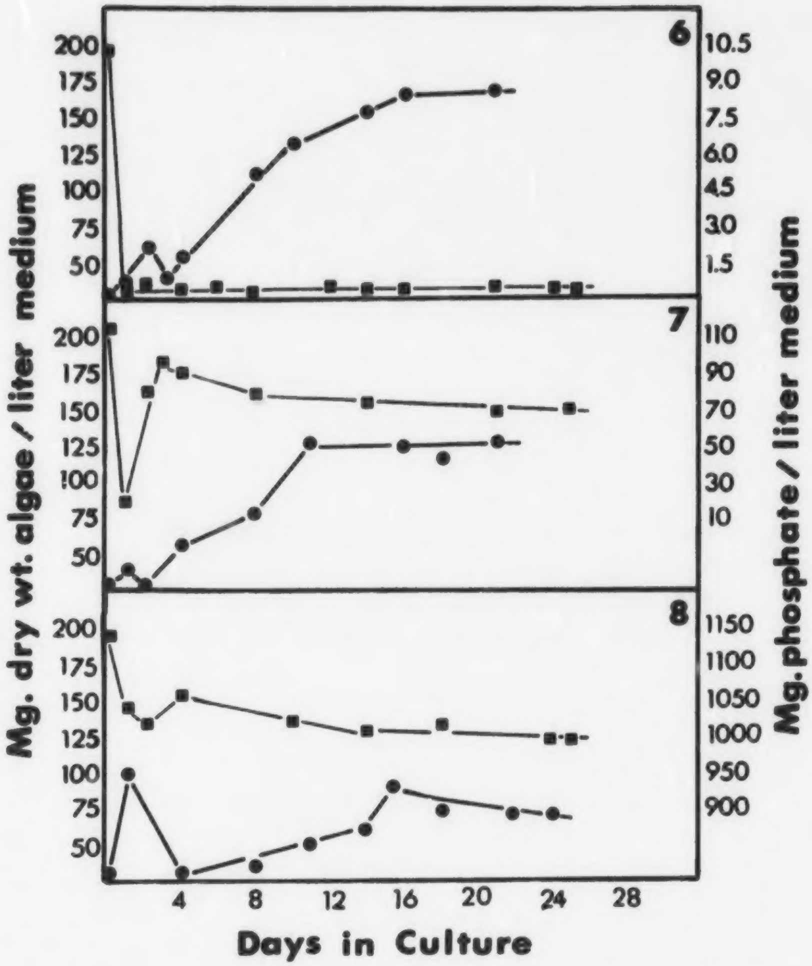
Oscillatoria tenuis was also cultured at the same phosphate concentrations as *P. boryanum*, but with quite different results. (Figures 9-15) *O. tenuis* did not grow well in phosphate concentrations above 10 mg PO_4 liter (Figures 14 and 15). At 1000 mg PO_4/1, *O. tenuis* increased in dry weight 2 days after transfer. However, the culture was not able to survive at this high phosphate concentration for more than 4 days (Figure 15). The phosphate depletion from the medium was similar to that of *P. boryanum* (Figures 9-15). *Plectonema boryanum* was then selected for all subsequent experiments due to its ability to tolerate a wide range of phosphate concentrations.

Figure 16 demonstrates the growth of the alga at 37°C. This rate was determined so that the alga could be acclimated to that temperature for uptake studies. By raising the temperature 12°C above room temperature, the growth rate was approximately doubled so that log phase occurred at 7 days. The 10 mg



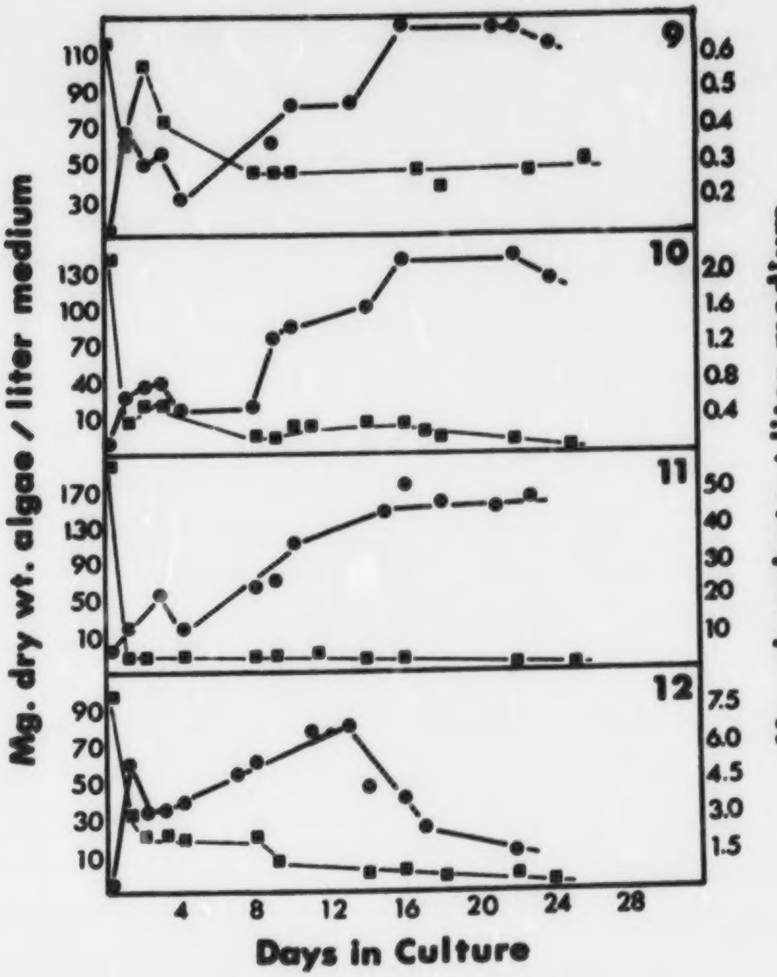
Figures 2-5. Growth curves of Plectonema boryanum and phosphate depletion of culture medium. All growth curves were determined at 25°C, 500 ft-candles of illumination, and a 12 hour alternating day/night cycle. Growth is represented by circles and phosphate depletion by squares.

Figure 2.	1 mg PO_4/ liter culture medium
Figure 3.	2 mg PO_4/ liter culture medium
Figure 4.	5 mg PO_4/ liter culture medium
Figure 5.	8 mg PO_4/ liter culture medium



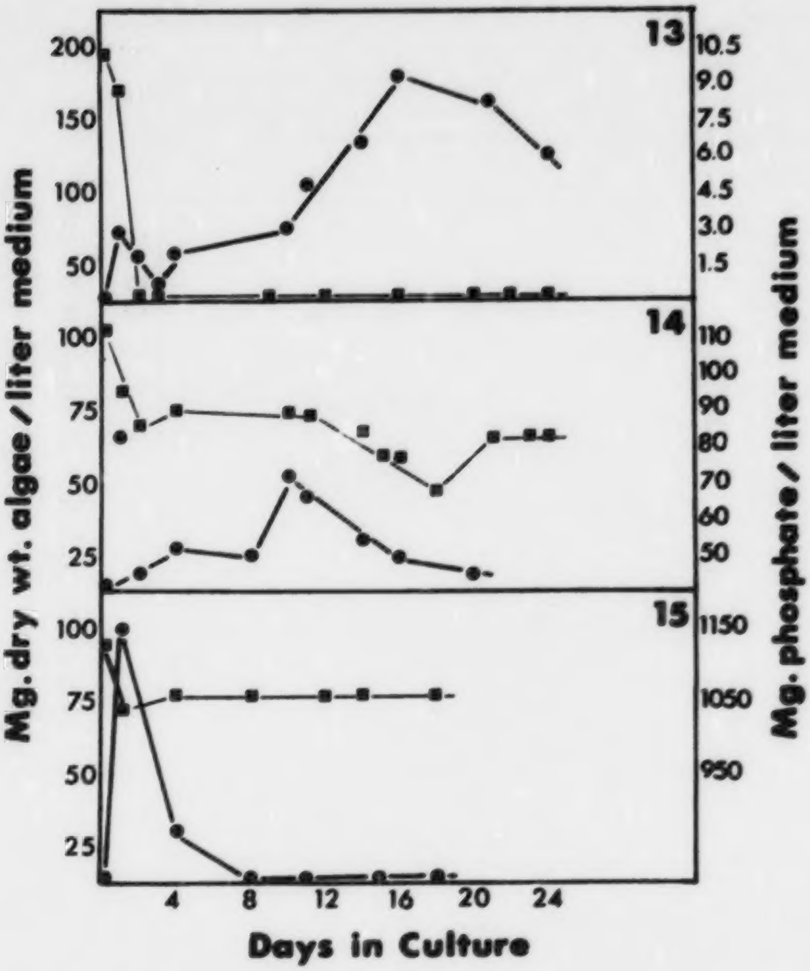
Figures 6-8. Growth curves of Plectonema boryanum and phosphate depletion of medium. All growth curves were determined at 500 ft-candles of illumination, 25° C, and 12 hour alternating day/night cycle. Growth is represented by circles and phosphate depletion of the medium by squares.

Figure 6. 10 mg PO_4/ liter
Figure 7. 100 mg PO_4/ liter
Figure 8. 1000 mg PO_4/ liter



Figures 9-12. Growth curves of Oscillatoria tenuis and phosphate of the culture medium. All growth curves were determined at 25°C, 500 ft-candles of illumination, and a 12 hour day/night cycle. Growth is represented by circles and phosphate depletion by squares.

Figure 9. 1 mg PO_4/ liter culture medium
Figure 10. 2 mg PO_4/ liter culture medium
Figure 11. 5 mg PO_4/ liter culture medium
Figure 12. 8 mg PO_4/ liter culture medium



Figures 13-15. Growth curves of Oscillatoria tenuis and phosphate depletion of the culture medium. All growth curves were determined at 25°C, 500 ft-candles of illumination, and a 12 hour alternating day/night cycle. Growth is represented by circles and phosphate depletion of the medium by squares.

Figure 13. 10 mg PO_4/ liter culture medium
Figure 14. 100 mg PO_4/ liter culture medium
Figure 15. 1000 mg PO_4/ liter culture medium

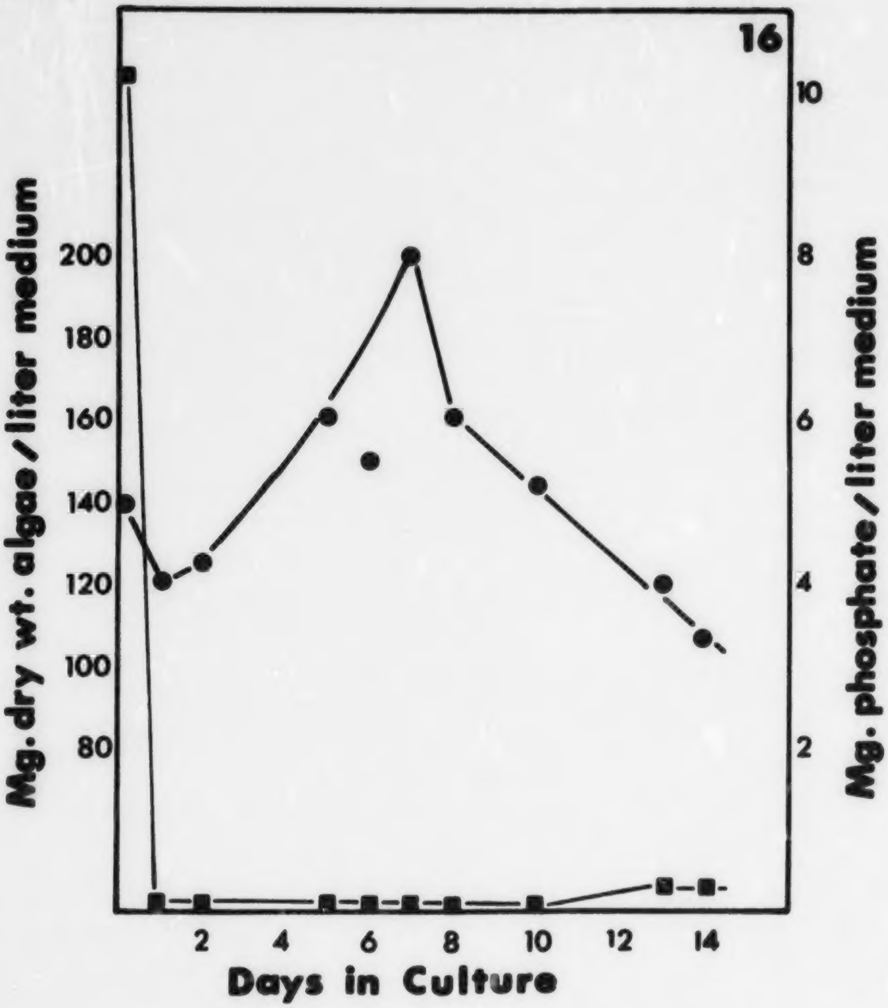


Figure 16. Growth curve of Plectonema boryanum and phosphate depletion of the culture medium. Growth conditions were 10 mg PO_4/ liter culture medium, 37°C, 500 ft-candles of illumination, and an alternating 12 hour day/night cycle. Growth is represented by circles and phosphate depletion of the culture medium by squares.

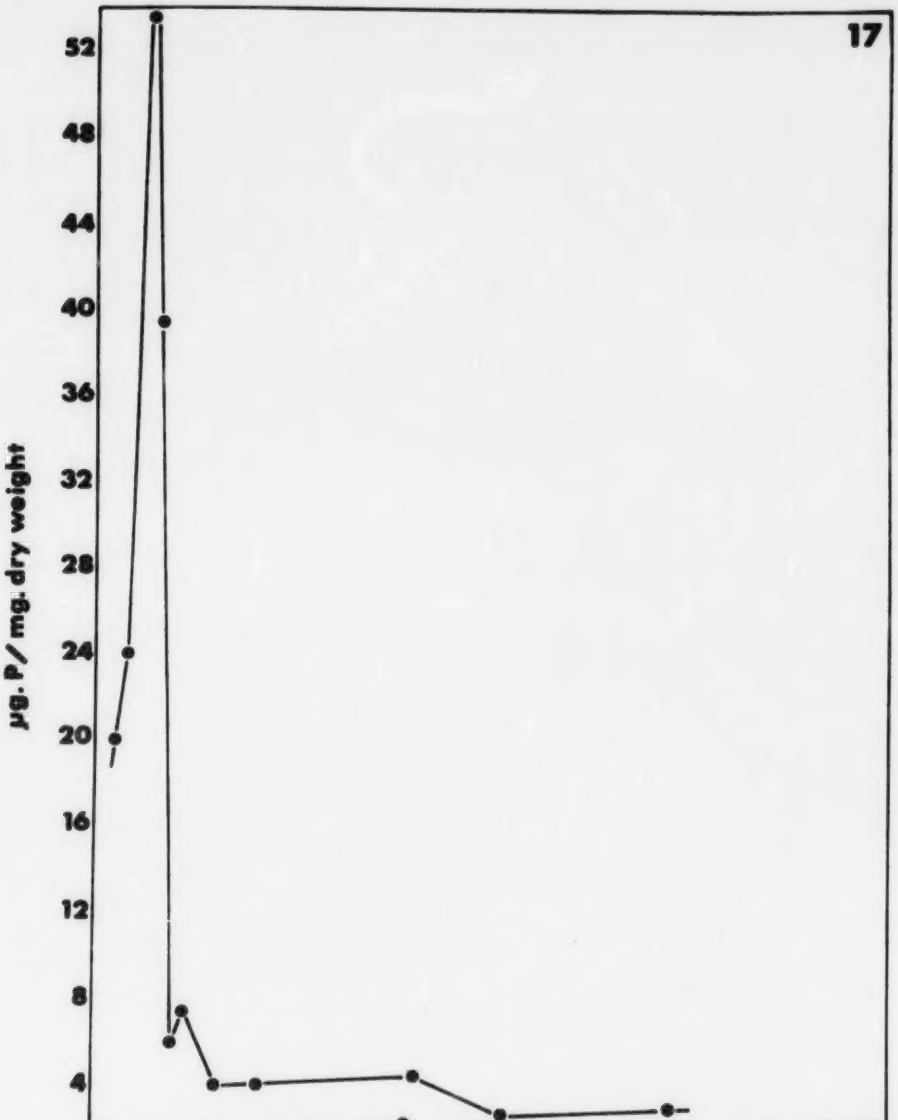


Figure 17. Total phosphorus Content of Plectonema boryanum as a function of culture age. The distribution is for cultures maintained at 25°C, and under the conditions as listed for Figures 3-9.

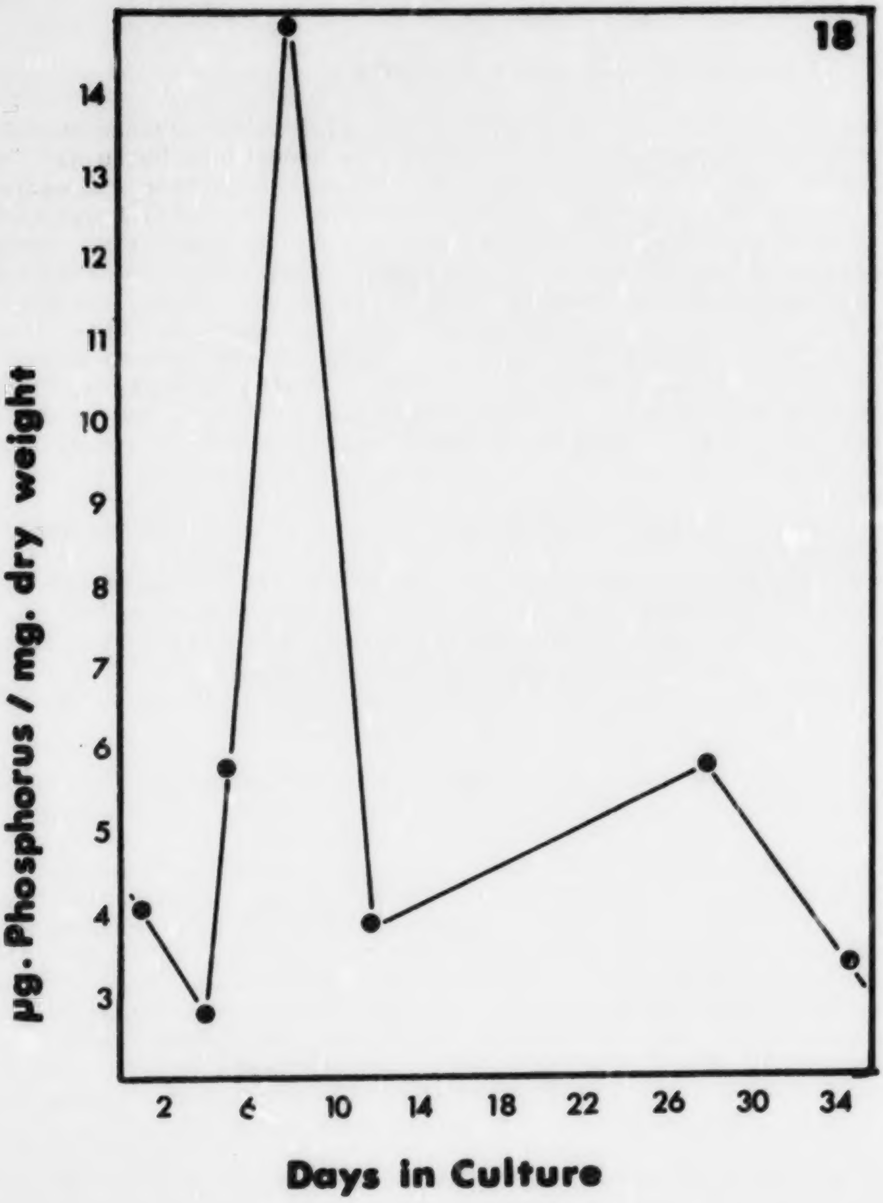


Figure 18. Total Phosphorus of P. *boryanum* as a function of culture age. Cultures were maintained at 37° C.

of phosphate initially present in the medium was depleted after 1 day of culture, and remained so for the 14 day period.

TOTAL PHOSPHATE PRESENT AS A FUNCTION OF GROWTH

The initial increase in dry weight of the alga upon transfer to fresh medium was of particular interest. It was initially thought to be an artifact, but it was also accompanied by a tremendous loss of phosphate from the medium and was present during all growth determinations. The total cell phosphorus was then measured for cultures of the alga between 1 and 51 weeks old. These results are presented in Figure 17. *P. boryanum*, inoculated into fresh Modified Fitzgerald medium containing between 7 and 10 mg PO_4 per liter, reaches a peak in total cell phosphorus at approximately 1 month of culture. This value is 53 µg P per mg dry weight algae (Figure 17). The cell phosphorus then declines and begins to attain a constant value at about 2 months of culture, this lower limit of phosphorus concentration being approximately 4 µg P per mg dry weight algae (Figure 17). Thus, transfers to fresh medium were always made from cultures which had reduced total cell phosphorus levels.

Similar values for total cell phosphorus levels were also determined for the 37°C cultures (Figure 18). It was found that the highest levels occurred on the seventh day after transfer of the cultures. This value was 14 µg P mg dry weight algae. The total cell phosphorus then declines to approximately 3.5 µg P per mg dry weight algae at the end of 34 days in culture. The lower values for the total cell phosphorus are similar for both the 25 and 37 cultures, while the maximum values are considerably higher in the 25°C cultures (Figures 17 and 18).

DETERMINATION OF OPTIMUM STARVATION LENGTH FOR RAPID UPTAKE FOR ALL THE FOLLOWING EXPERIMENTS

Logarithmic phase cells were used for all following experiments. Cells in this metabolic state were chosen because the amount of polyphosphate present should be at its lowest level (refer to Aspects of Phosphate Metabolism Section of Literature Review). Since the length of time necessary to deplete the cell of any phosphorus reserves was not known, a series of experiments were set up to determine the effects of phosphate starvation on rates of phosphate uptake. Cells taken from logarithmic phase were inoculated into phosphate-free medium for 3, 5, 11, and 12 days. The cells were then reinoculated into medium containing a known amount of phosphate, and the cells were then analyzed for total phosphorus content. These results are presented in Figure 19.

Phosphate uptake is represented by increase in total cell phosphorus for these experiments. Virtually no uptake occurred after 3 days of starvation (see legend to Figure 19 for environmental parameters). After 5 days of starvation, total cell phosphorus at the end of the uptake period increased from approximately 5 to 50 µg phosphorus per mg dry weight algae. Prolonging the starvation period to 12 days had no significant effect on the uptake rate. Starvation of the algae for 11 days and subsequent inoculation into a lower phosphate concentration (10 mg PO_4 per liter as opposed to 92.50 mg PO_4 per liter as above) resulted in uptake, but with reduced magnitude. Thus, 5 days of incubation in phosphate-free medium at 25°C, 500 ft-candles of illumination, and

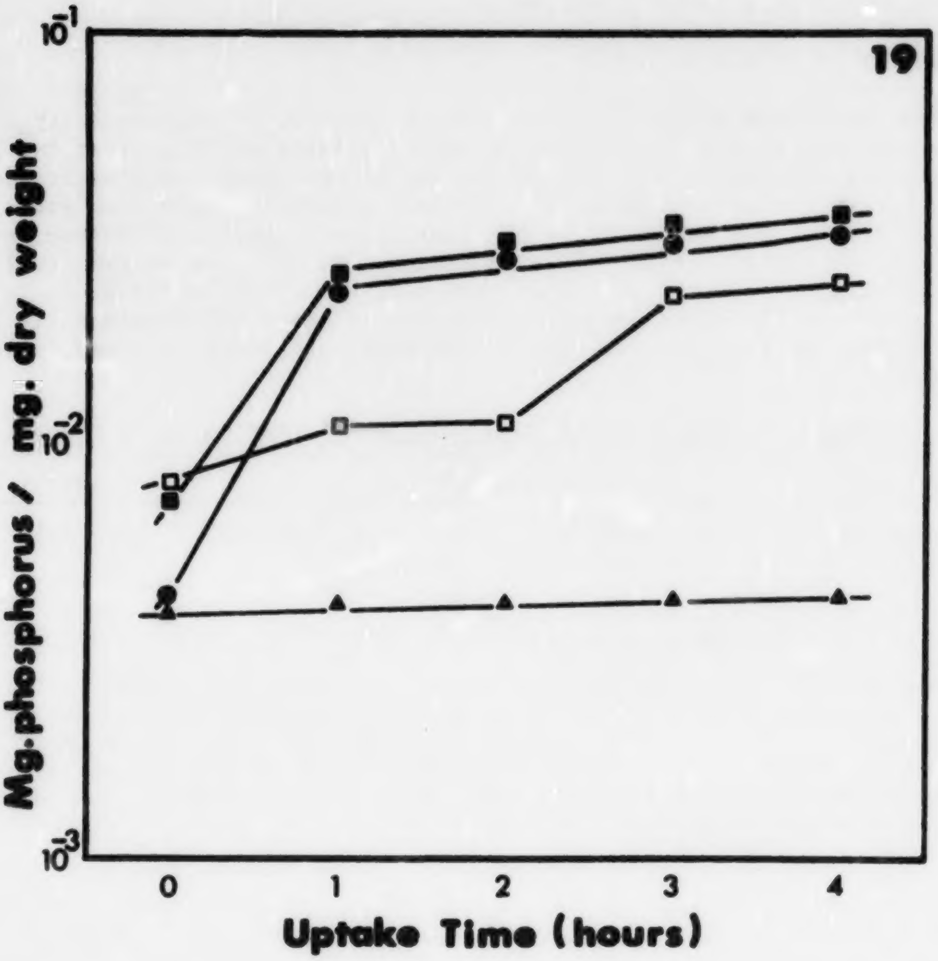


Figure 19. Semi-logarithmic plot of increase in total cell phosphorus as a function of increasing the length of time in phosphate-free medium (starvation). All uptakes were at 25°C, and 500 ft-candles of illumination. Closed squares represent 12 days of starvation and subsequent inoculation into medium containing 92.51 mg PO_4/ liter.

Closed Circles represent 5 days of starvation and uptake from 92.51 mg PO_4/ liter.

Open Squares represent 11 days of starvation and uptake from 10 mg PO_4/ liter.

Closed Triangles represent 3 days of starvation and uptake from 10 mg PO_4/ liter.

a 12 hour alternating day/night cycle was chosen to be the minimum time length necessary to induce phosphate starvation.

DETERMINATION OF UPTAKE PARAMETERS

After the determination of starvation conditions, it was necessary to select parameters to monitor for the uptake of phosphate. The first two physical parameters considered were the dry weight of the algae and loss of phosphate from the medium (refer to Figure 21). Orthophosphate in the medium was essentially depleted at the end of the 4 hours of uptake. During this same period of time, the dry weight of the algae increased. Studies correlating cell number and dry weight analysis of normal, starved, and uptake cells indicate that the dry weight of the cells decreases during starvation and subsequently increases during rapid uptake, but not to the levels of normally grown cells (Table 2).

TABLE 2. CHANGES IN CELL SIZE AND WEIGHT DURING STARVATION AND RAPID UPTAKE

Cell Size (µm)	Dry Weight(mg)	Average Wt. Cell	Cells/Mg Dry Wt.
Normal 4.01 ± 1.93	330	1.98×10^{-7} mg/cell	5.5×10^{6}
Starved 2.87 ± 0.73	60	2.20×10^{8}	
Uptake 2.80 ± 9.69	200	7.48×10^{-8}	

The average cell size decreased during starvation and uptake, indicating unusual and/or rapid cell division. During the starvation period, the average weight of the cell decreased by an order of magnitude. This value increased during uptake, but not to the value of a normal cell. These values were obtained by counting 300 filaments averaging 8 cells per filament, and taking dry weight measurements of the culture. For most cultures, 1 mg of dry weight corresponds to 5.5×10^{6} cells.

Figure 20 demonstrates that during the four hour uptake period, the largest increase in total cell phosphorus and cold TCA extractable phosphorus occurs at 1 hour. The total cell phosphorus increases from about 8 to 13 µg P/mg dry weight and the cold TCA extractable phosphorus increases from 1 to 7 µg P/mg dry weight algae. This uptake then begins to level off, and is virtually complete at four hours. It was for these reasons that uptake was usually monitored for a four hour period.

PHYSICAL PARAMETERS AFFECTING PHOSPHATE UPTAKE RATES

Temperature

Rapid uptake of orthophosphate by the algae was monitored at three different temperatures, 4, 25, and 37° C. The 25 and 37°C uptakes were carried out on algae acclimated to those respective temperatures as previously described. Since the algae did not grow at 4° C, they were grown at 25 , and chilled for 1 hour prior to inoculation into a phosphate-containing medium. These results are presented in Figure 22. This graph demonstrates that there was little uptake at 4° C, and a definite reduction in total cell phosphorus after 2 hours of incubabation at this temperature. The uptake response at 37 is essentially the same as that of 25°C. This is unlike the growth rate response at 37°C, which was a doubling.

Light

For all rapid uptakes at different light intensities, the algae were incubated during the starvation period at 300 ft-candles of illumination and then incubated at different light intensities for the uptake. Variations in light intensity were accomplished by placing the culture flasks at different levels from the light source in the growth chamber, or in the case of total darkness, by covering the flasks entirely with aluminum foil. Figure 23 shows the results of these experiments. In total darkness or at 100 ft-candles, there was little uptake of orthophosphate, as expressed by increase in total cell phosphorus. The total cell phosphorus increased when the algae were exposed to orthophosphate at 500, 1000, and 2000 ft-candles of illumination. This increase was directly related to the light intensity. The greatest increase in total cell phosphorus for all light intensities occurred within the first hour of uptake (Figure 23).

Effect of Phosphate Concentration on Uptake

Monitoring the phosphate depletion from the medium led to studies which were designed to test the maximum amount of phosphate that could be utilized by the algae. It was found that the algae could essentially deplete the medium of phosphorus if given limited amounts (Figures 21, 24, and 25). These uptakes were carried out in medium containing 10 mg PO_4 (3.26 mg P) per liter or less. By increasing the amount of orthophosphate available in the medium to 92.51 mg per liter, the algae removed more phosphate from the medium, and the total cell phosphorus of the algae also increased (Figure 26). These results are summarized in Table 3.

Effects of Inhibitors on Uptake

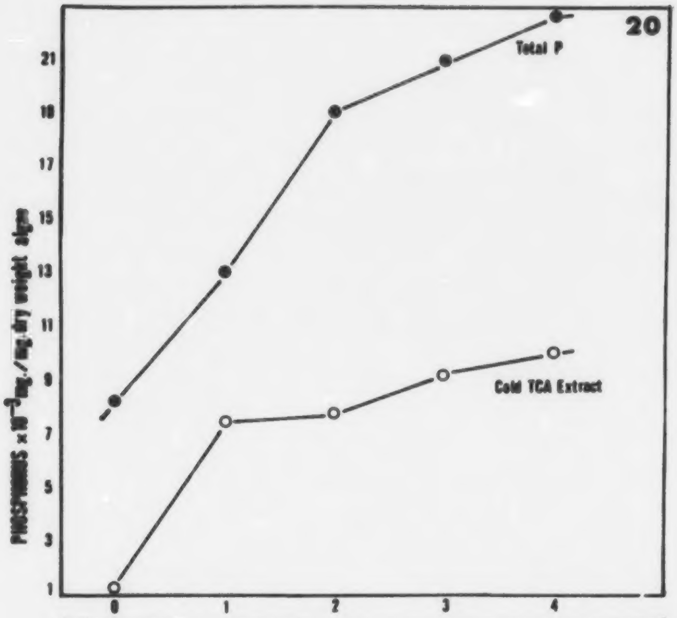
For all studies of inhibitors, the algae were pre-incubated with the inhibitor for one hour prior to the addition of phosphate. Figure 27 illustrates the results of these experiments. There was virtually no uptake when 1×10^{-3} sodium fluoride, 1×10^{-2} mercuric chloride, or 5×10^{-3} 2,4-dinitrophenol were used as inhibitors. Of the three tested, sodium fluoride was the least effective. The upper curve in Figure 27, an uptake with no inhibitors present, under otherwise identical conditions, is plotted as a reference curve.

Phosphate Uptake from Starvation Period with .01 mg PO_4/1

Figures 28 and 29 show that even when cells are starved for 5 days in culture medium with .01 mg PO_4/1 a rapid uptake is induced in the cells. The rate and amount of uptake is similar to that of cells starved in PO_4 free medium.

pH Effect on Uptake

Figures 30 and 31 show that uptake is very pH dependent. Very little phosphate is taken up at pH 7 but at pH 9 there is considerable uptake. When starved cells at pH 9 are placed in medium containing 100 mg PO_4/1 they can reduce the



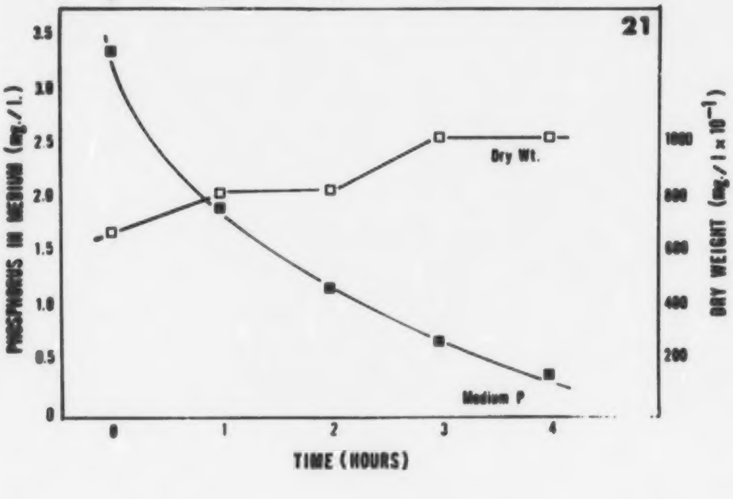


Figure 20. Increase in total cell phosphorus and cold TCA extractable phosphorus during rapid uptake at 25°C, 500 ft-candles of illumination and 10 mg PO_4/liter.

Figure 21. Increase in dry weight of the algae and depletion of medium phosphorus during rapid uptake as described for Figure 20.

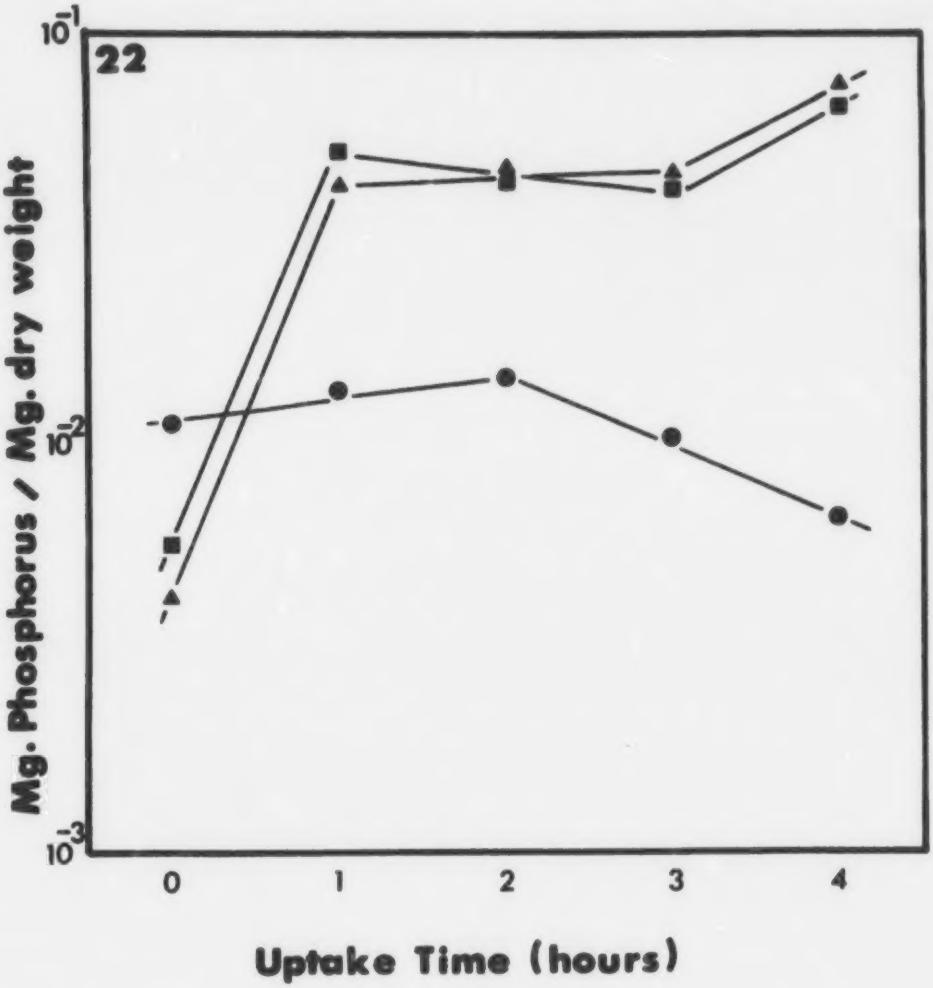


Figure 22. Semi-logarithmic plot of the increase in total cell phosphorus vs. uptake time as a function of temperature.

Triangles represent uptake at 25°C.

Squares represent uptake at 37°C.

Circles represent uptake at 4°C.

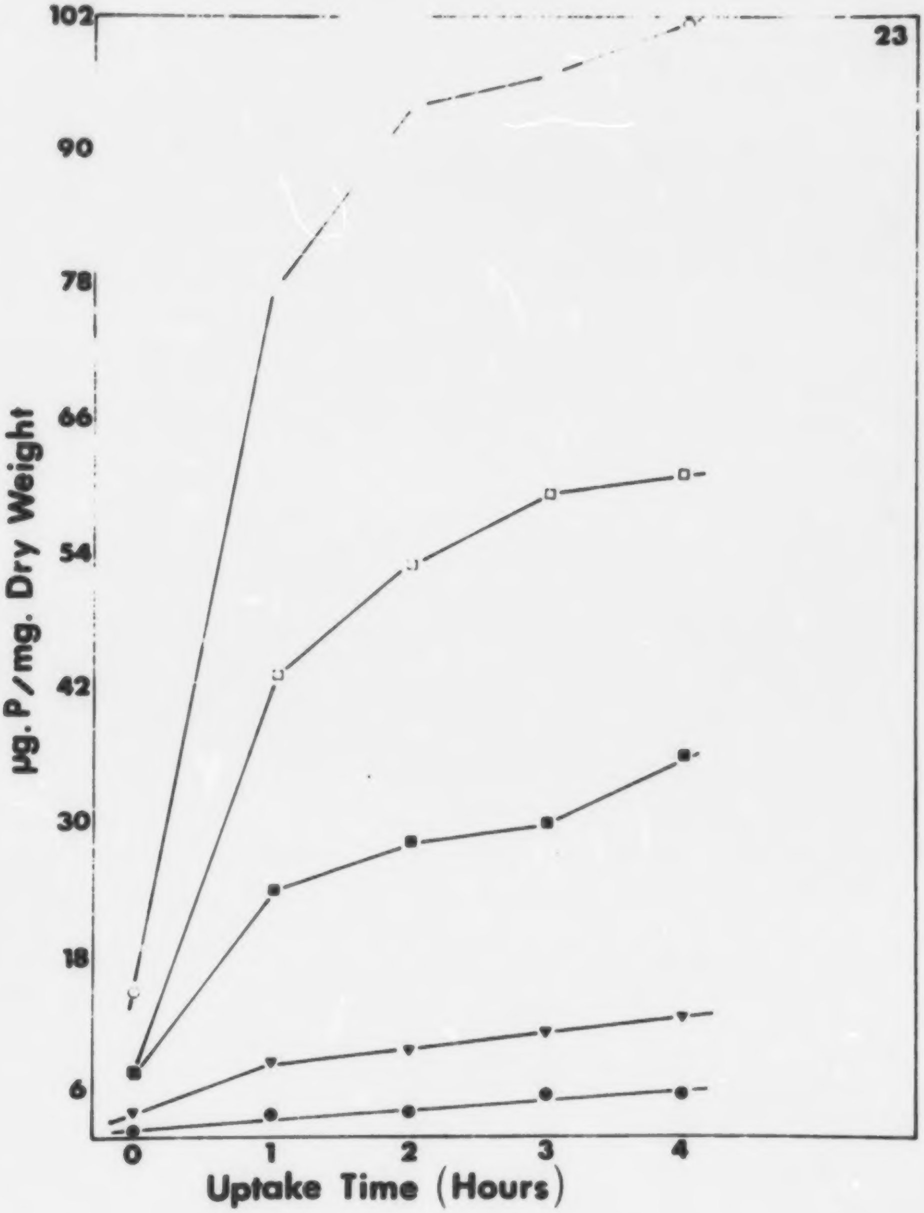
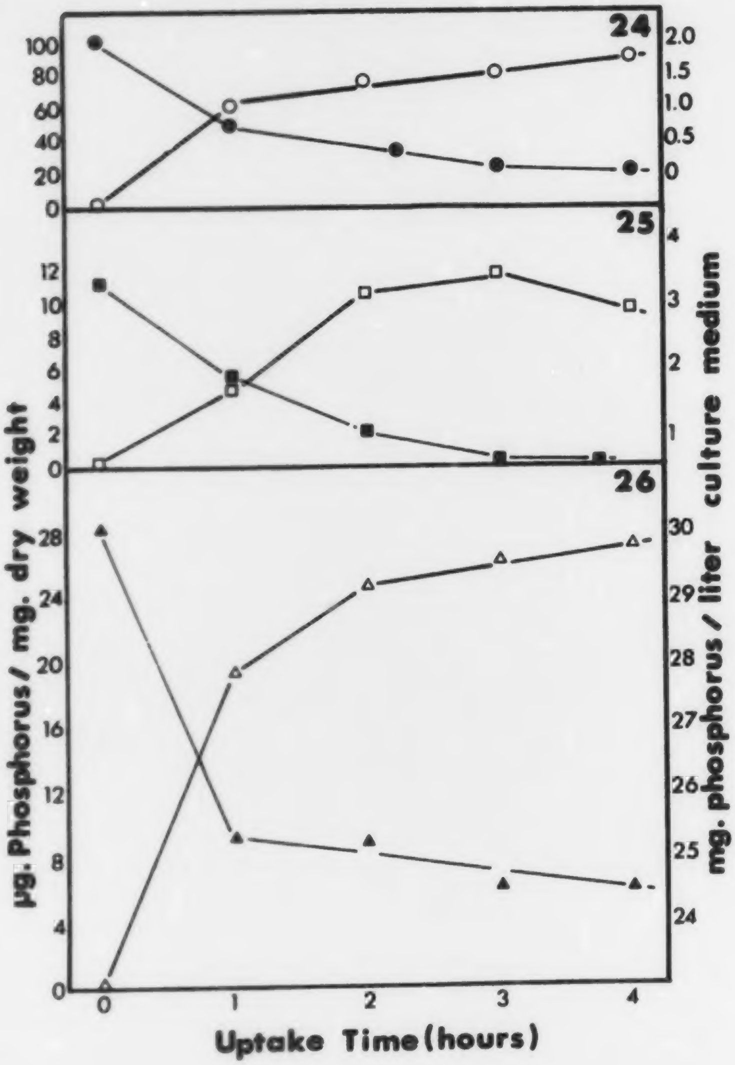


Figure 23. Increase in total cell phosphorus during rapid uptake as a function of light intensity. The graphs from top to bottom, represent 2000, 1000, 500, 100, and 0 ft-candles of illumination.



Figures 24-26. Change in total cell phosphorus and phosphate depletion of the medium as a function of orthophosphate concentration in the medium. Total cell phosphorus is represented by open symbols, and phosphate depletion of the medium by closed symbols.

Figure 24. 6.58 mg PO_4/ liter culture medium
Figure 25. 10.13 mg PO_4/ liter culture medium
Figure 26. 92.51 mg PO_4/ liter culture medium

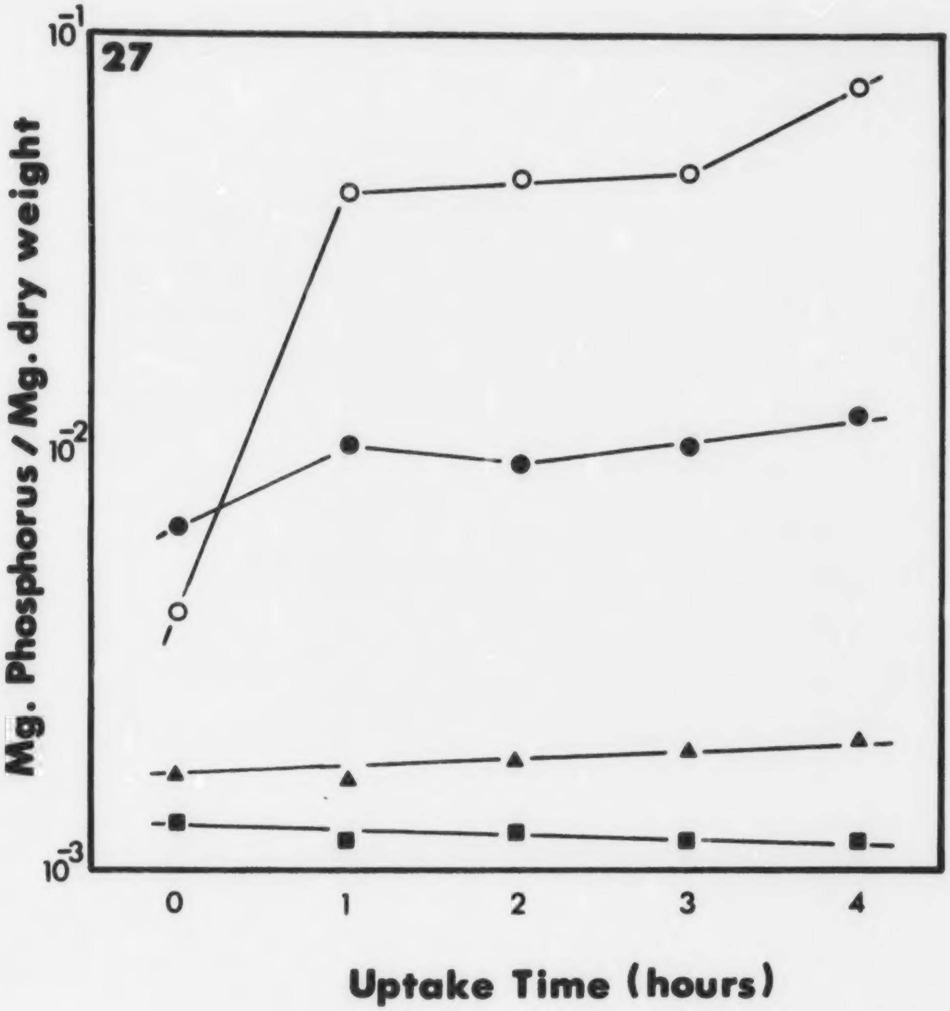


Figure 27. Effect of metabolic inhibitors on the rapid uptake process. Semilogarithmic plot of increase in total cell phosphorus vs. time. Variation of phosphate levels at time 0 can be explained by the standard deviations encountered during sampling.

Open Circles represent a normal uptake.

Closed Circles represent 1×10^{-3} M sodium fluoride

Closed Triangles represent 1×10^{-2} M mercuric chloride

Closed Squares represent 5×10^{-3} M 2,4-dinitrophenol

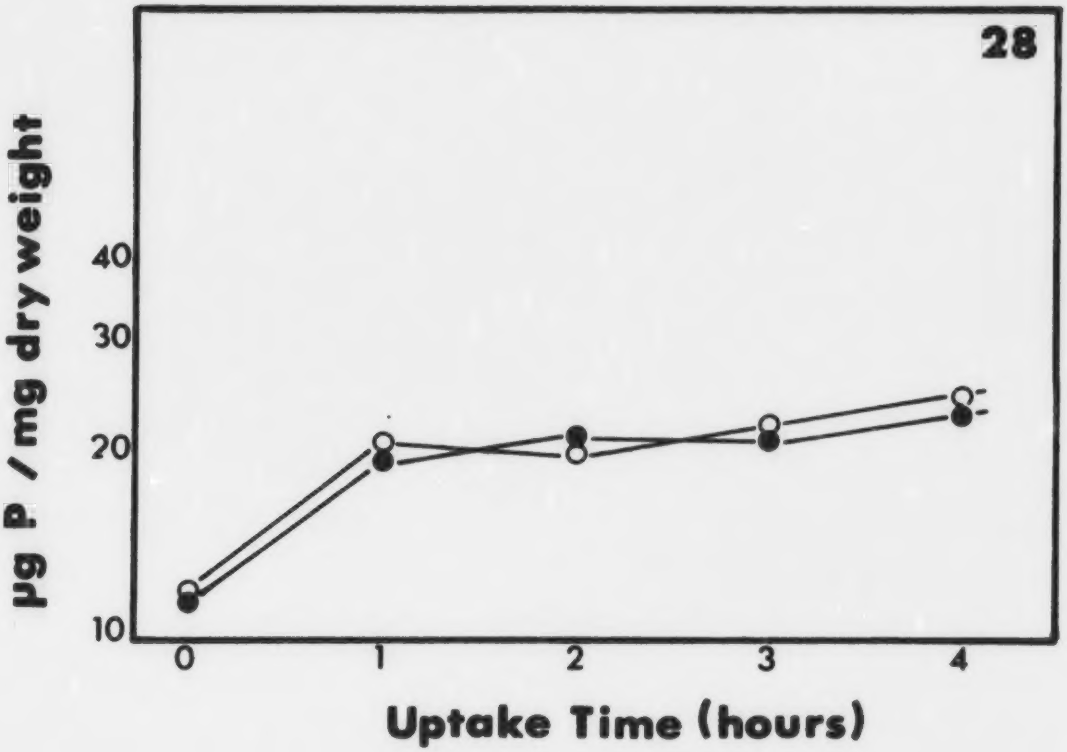


Figure 28. Semi-logarithmic plot of total phosphorus in cells after rapid uptake in 10 mg PO_4/l. Cells were starved at 500 ft-candles, 75°F for 5 days in medium containing 0.01 mg PO_4/l. Medium replenished daily. Rapid uptake done at 500 ft-candles at 75°F. Open and closed circles represent separate experiments.

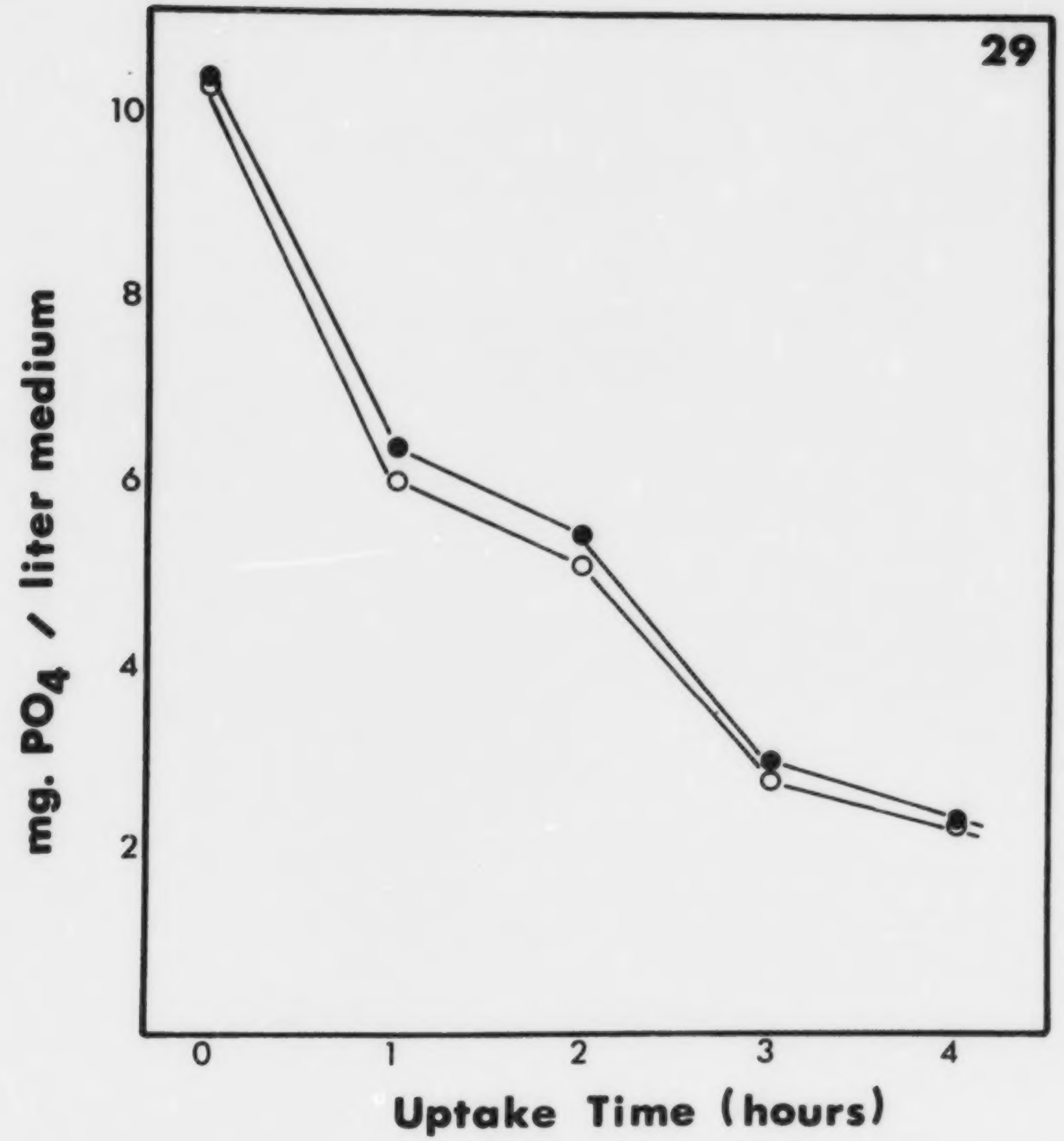


Figure 29. Phosphorus left in the medium during rapid uptake by cells which were starved of phosphorus for 5 days at 500 ft-candles, 75°F in medium containing 0.01 mg PO_4/1. Medium replenished daily. Open and closed circles represent separate experiments.

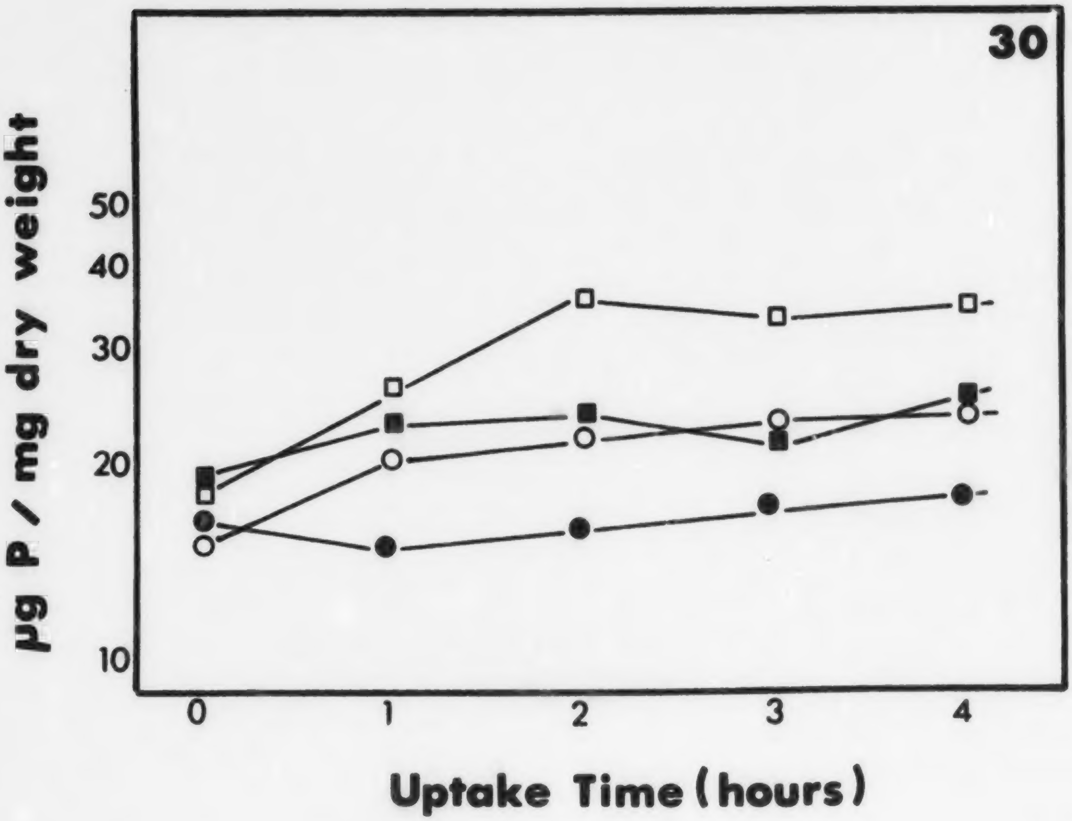


Figure 30. Total phosphorus in cells after rapid uptake at pH 7 in 10 mg PO_4/1 (●) and 100 mg PO_4/1 (○). Rapid uptake done at pH 9 in 10 mg PO_4/1 (■) and 100 mg PO_4/1 (□). Cells grown and starved of phosphorus under normal culture conditions. Semi-log plot.

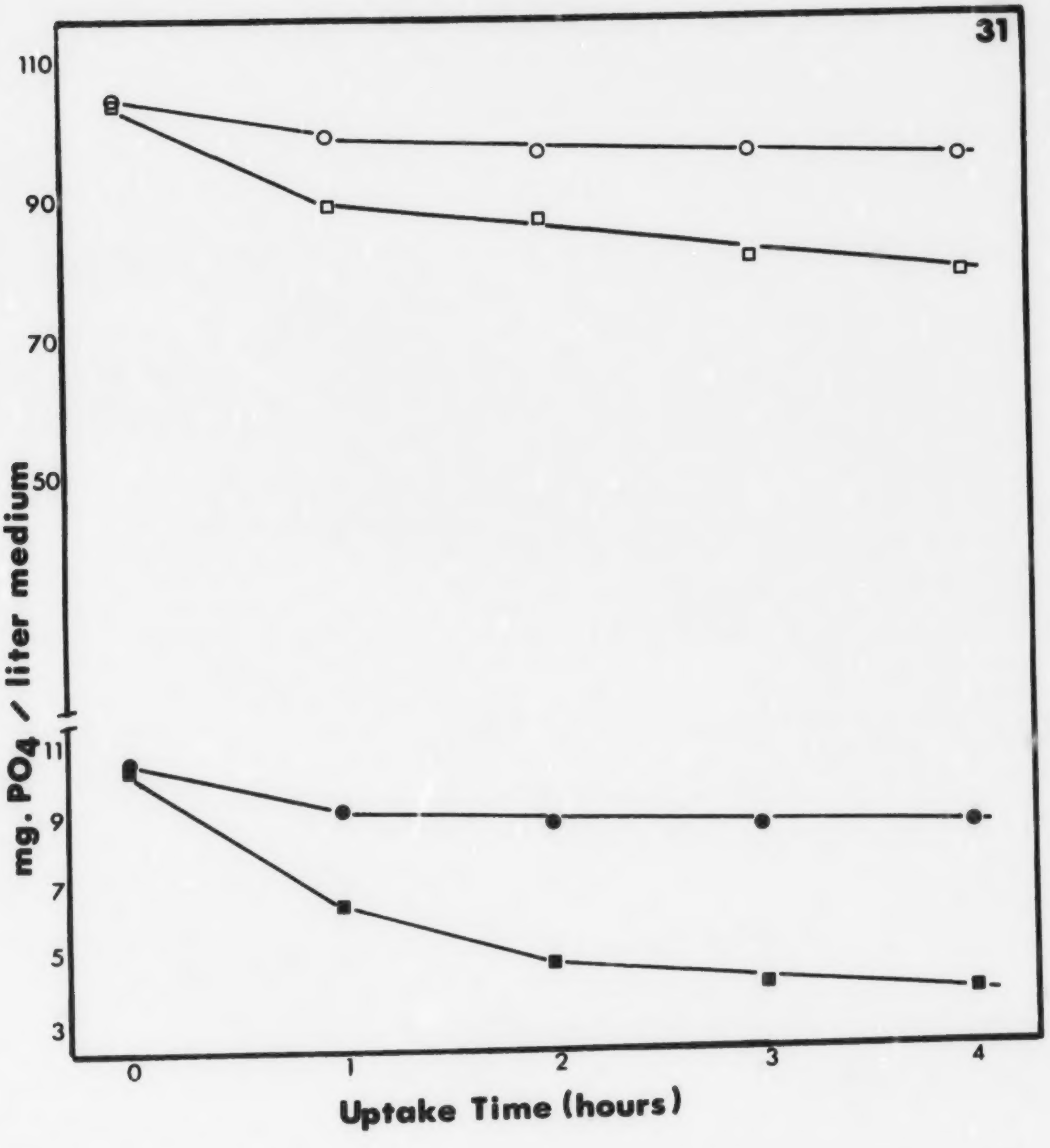


Figure 31. Phosphorus left in the medium after 4 hrs of rapid uptake at pH 7 and pH 9. Rapid uptake done at pH 7 in 10 mg PO_4/1 (●) and 100 mg PO_4/1 (○). Rapid uptake done at pH in 10 mg PO_4/1 (■) and 100 mg PO_4/1 (□).

level more than 10 mg PO_4/1.

Uptake From Distilled Water--Ion Effects

Figures 32-39 show the effect of ions in distilled water on rapid uptake. When the phosphorus left in the medium is monitored it appears in several cases that considerable uptake has occurred. However, when total cell phosphorus is determined little uptake is revealed. This is probably due to some sort of differential trapping of the phosphate in the sheath under these conditions. Thus it would not be present in the culture medium alone and could be washed out during preparation of the samples for a total phosphorus determination.

As can be seen from this data at a pH of 7 virtually no uptake occurs with any of the ions tested (Figures 32 and 33). At pH 9 potassium, calcium, sodium and magnesium either alone or in combination with one other ion seems to be necessary for uptake (Figure 34). However, in no case does the uptake equal that of the complete medium. Figure 35 shows that with sodium, calcium and magnesium present the greatest uptake is found with various combinations of these ions and the greatest uptake with all present.

Effect of Starvation of Phosphate and Ions

Figures 40-54 show the effect of phosphate and ion starvation on the uptake of phosphorus in the absence of certain ions. These results indicate that as long as potassium, calcium or magnesium are present either alone or in combination the rapid uptake will occur. Some of the data however indicate that calcium may be of special importance (Figures 41, 50 and 53). Magnesium does not seem to be critical for uptake (Figure 42). Table 4 shows the concentration of the various ingredients in Fitzgerald's modified medium.

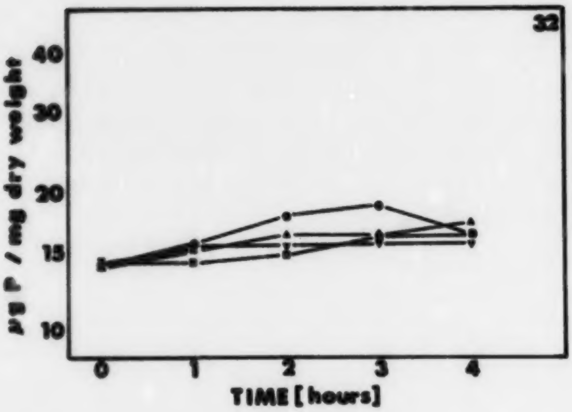
Effect of High Ionic Concentration During Starvation and Uptake

Figures 55-57 show the effect of a high ionic concentration during starvation and during rapid uptake. Figures 55 and 56 demonstrate that a high ionic concentration of mg and K have no effect on total amount of phosphate assimilated. However, calcium in high concentrations enhance the uptake of phosphorus (Figure 57).

NORMAL LEVELS OF PHOSPHORUS-CONTAINING COMPOUNDS IN *PLECTONEMA BORYANUM*

After preliminary data were obtained about the "overplus" phenomenon in *P. boryanum*, studies were conducted to determine the distribution of phosphorus in various extracts. Nucleic acids as determined in each of these fractions, and determined for whole cell digests was no greater than 10^{-6} mg P per mg dry weight in any case, which is considerably less than any fraction reported. It is for this reason that they are not included in the data.

P. boryanum was grown for 14 days to logarithmic phase under normal culture conditions in Modified Fitzgerald containing various concentrations of phosphate. The algae were then extracted as previously described, and the extracts were measured for total phosphorus concentration. These results are presented in Table 5. Average values for triplicate samples, varying no



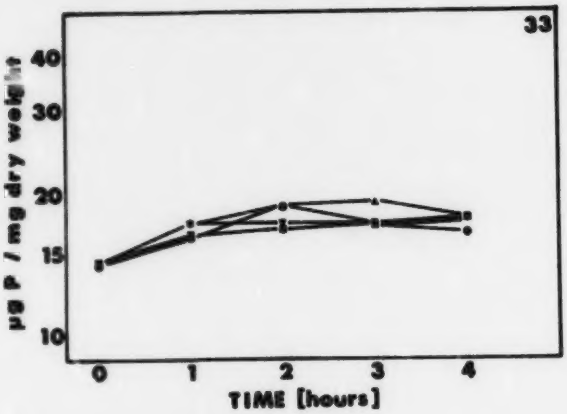
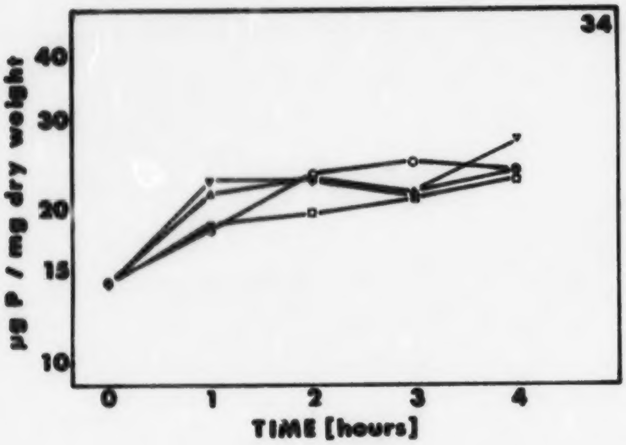


Figure 32. Total phosphorus in cells during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 7. Uptake done at 500 ft-candles, 75°F, pH 7 (adjusted with HCl) in 300 ml of distilled water containing K^+ (●), K^+ and Ca^{++} (▲), K^+ and Mg^{++} (■) and K^+, Ca^{++} and Mg^{++} (▼). Ions added at same concentration as in Fitzgerald's modified medium. Semi-log plot.

Figure 33. Total phosphorus in cells during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 7. Uptake done at 500 ft-candles, 75°F, pH 7 (adjusted with HCl) in 300 ml of distilled water containing Na^+ (●), Na^+ and Ca^{++} (▲), Na^+ and Mg^{++} (■), and Na^+, Ca^{++} and Mg^{++} (▼). Ions added in same concentration as in Fitzgerald's modified medium except Na^+ was kept at the same level as K^+. Semi-log plot.



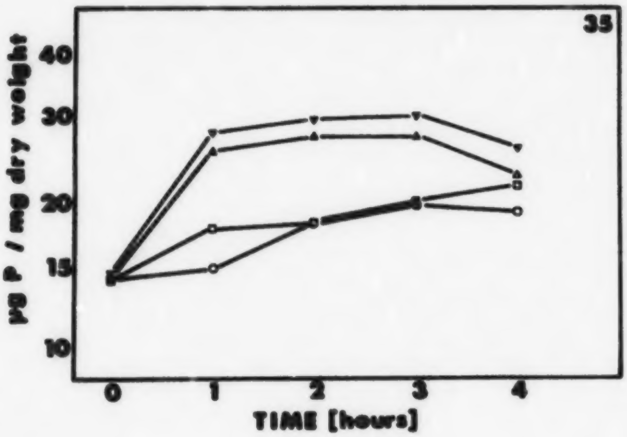


Figure 34. Total phosphorus in cells during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 9. Uptake done at 500 ft-candles, 75°F, pH 9 (adjusted with NaOH) in 300 ml of distilled water containing K^+ (○), K^+ and Ca^{++} (△), K^+ and Mg^{++} (□) and K^+, Ca^{++} and Mg^{++} (▽). Ions added in same concentration as in Fitzgerald's modified medium. Semi-log plot.

Figure 35. Total phosphorus in cells during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 9. Uptake done at 500 ft-candles, 75°F, pH 9 (adjusted with NaOH) in 300 ml of distilled water containing 10 mg PO_4/1 and Na^+ (○) Na^+ and Ca^{++} (△) Na^+ and Mg^{++} (□), and Na^+, Ca^{++} and Mg^{++} (▽). Ions added in same concentration as in Fitzgerald's modified medium except Na^+ was kept at the same level as K^+. Semi-log plot.

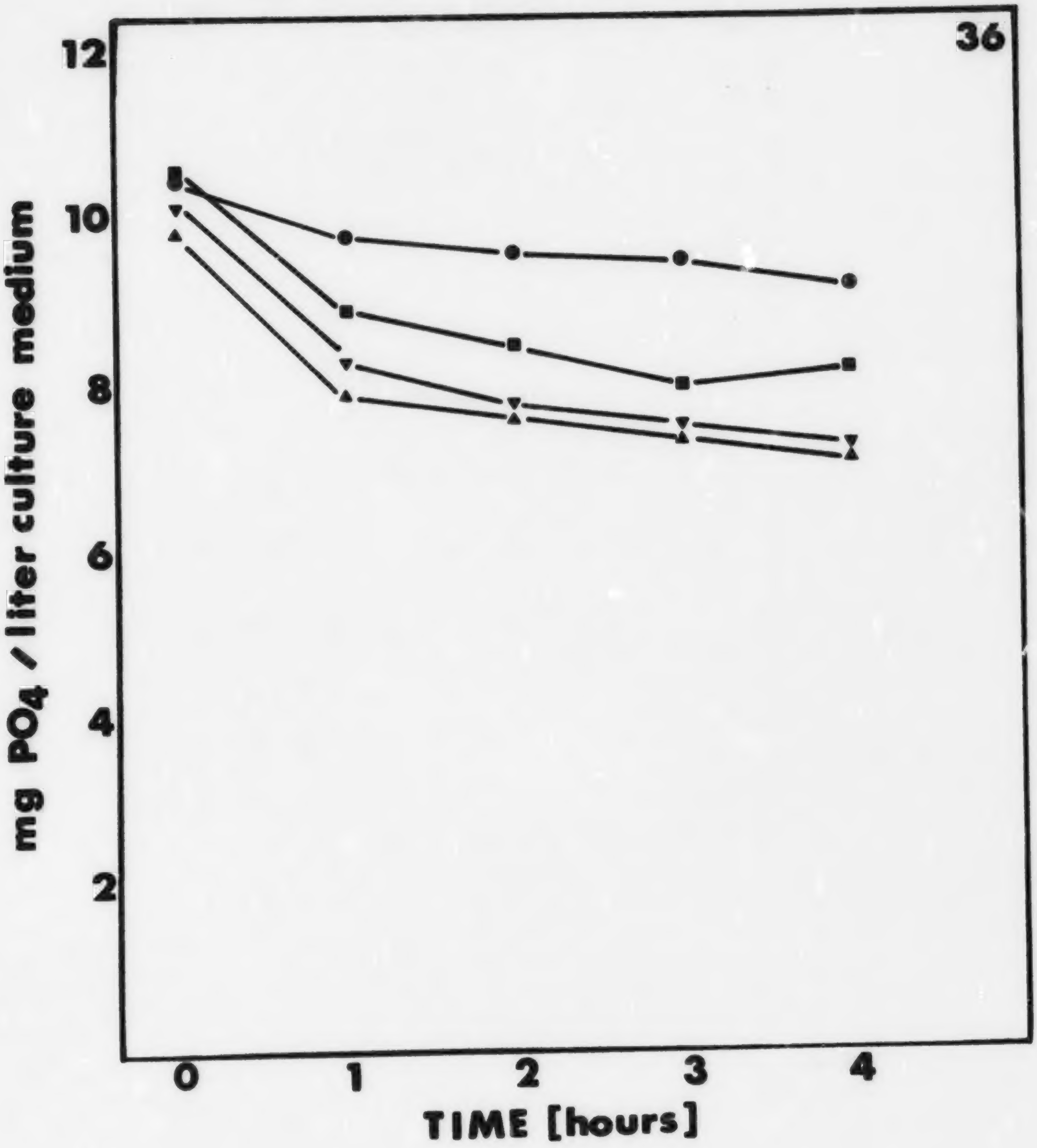


Figure 36. Phosphorus left in the medium during rapid uptake by cells starved in phosphate free medium for 5 days at a pH of 7. Uptake done at 500 ft-candles, 75°F in 300 ml of distilled water containing 10 mg PO_4/1 and K^+ (●), K^+ and Ca^{++} (▲), K^+ and Mg^{++} (■), and K^+, Ca^{++} and Mg^{++} (▼). Ions added in same concentration as in Fitzgerald's modified medium.

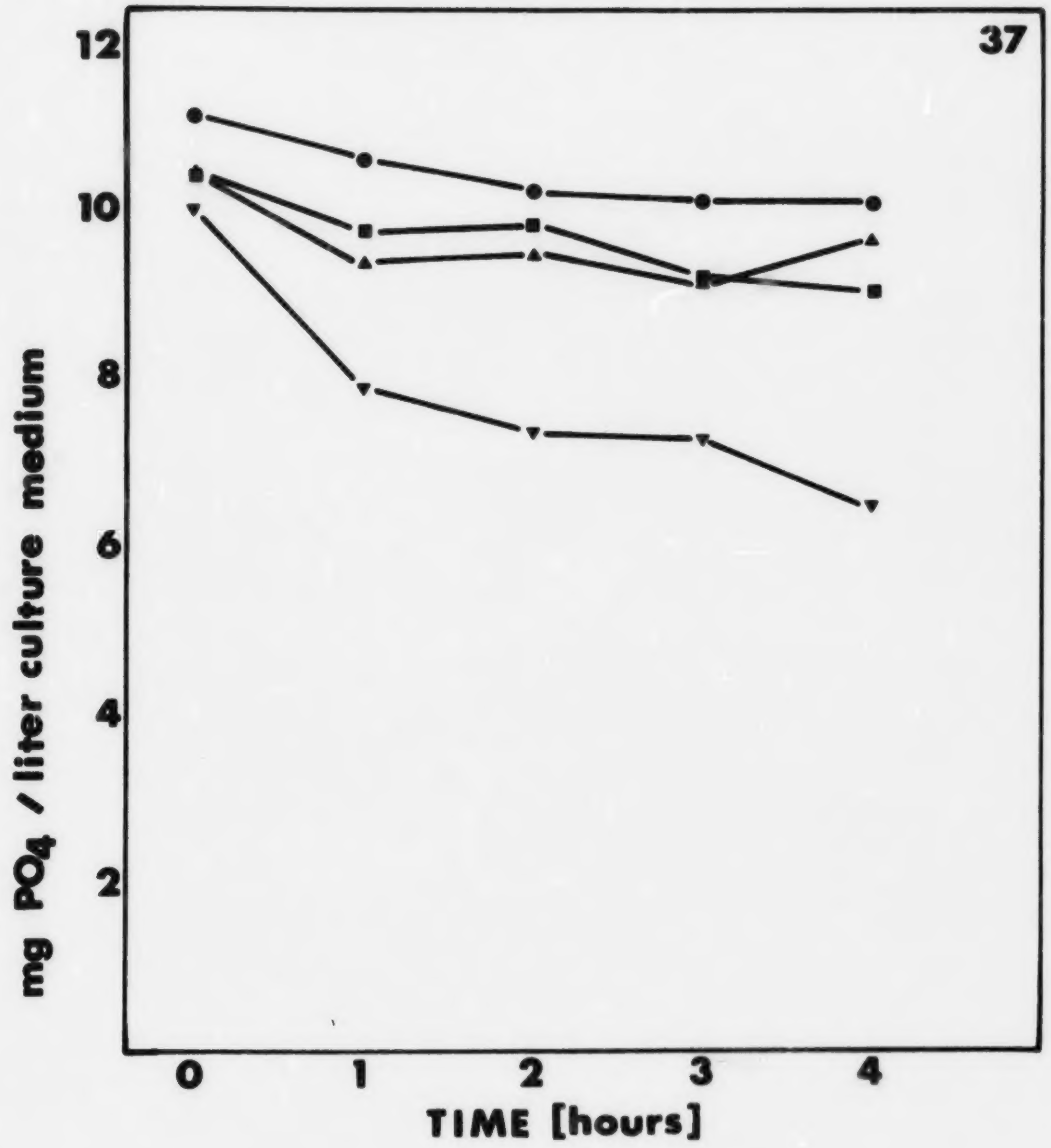


Figure 37. Phosphorus left in the medium during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 7. Uptake done at 500 ft-candles, 75°F and in 300 ml of distilled water containing 10 mg PO_4/l and Na^+ (●), Na^+ and Ca^{++} (▲), Na^+ and Mg^{++} (■) and Na^+, Ca^{++} and Mg^{++} (▼). Ions added in same concentrations as in Fitzgerald's modified medium except Na^+ is at same level as K^+.

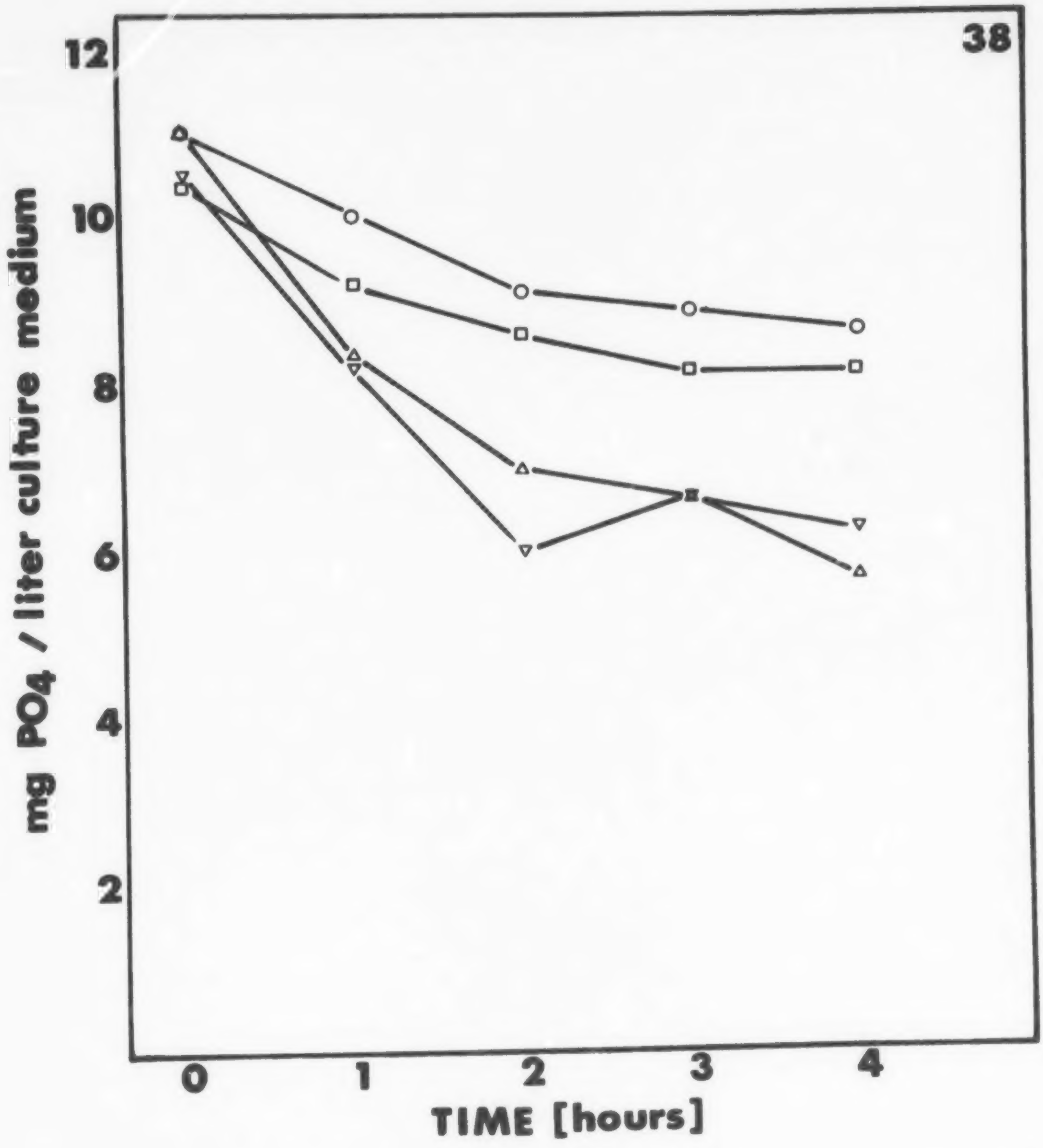


Figure 38. Phosphorus left in the medium during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 9. Uptake done at 500 ft-candles, 75°F, pH 9 (adjusted with NaOH) in 300 ml of distilled water containing 10 mg PO_4/1 and K^+ (○), K^+ and Ca^{++} (△), K^+ and Mg^{++} (□), and K^+, Ca^{++} and Mg^{++} (▽). Ions added in same concentration as in Fitzgerald's modified medium.

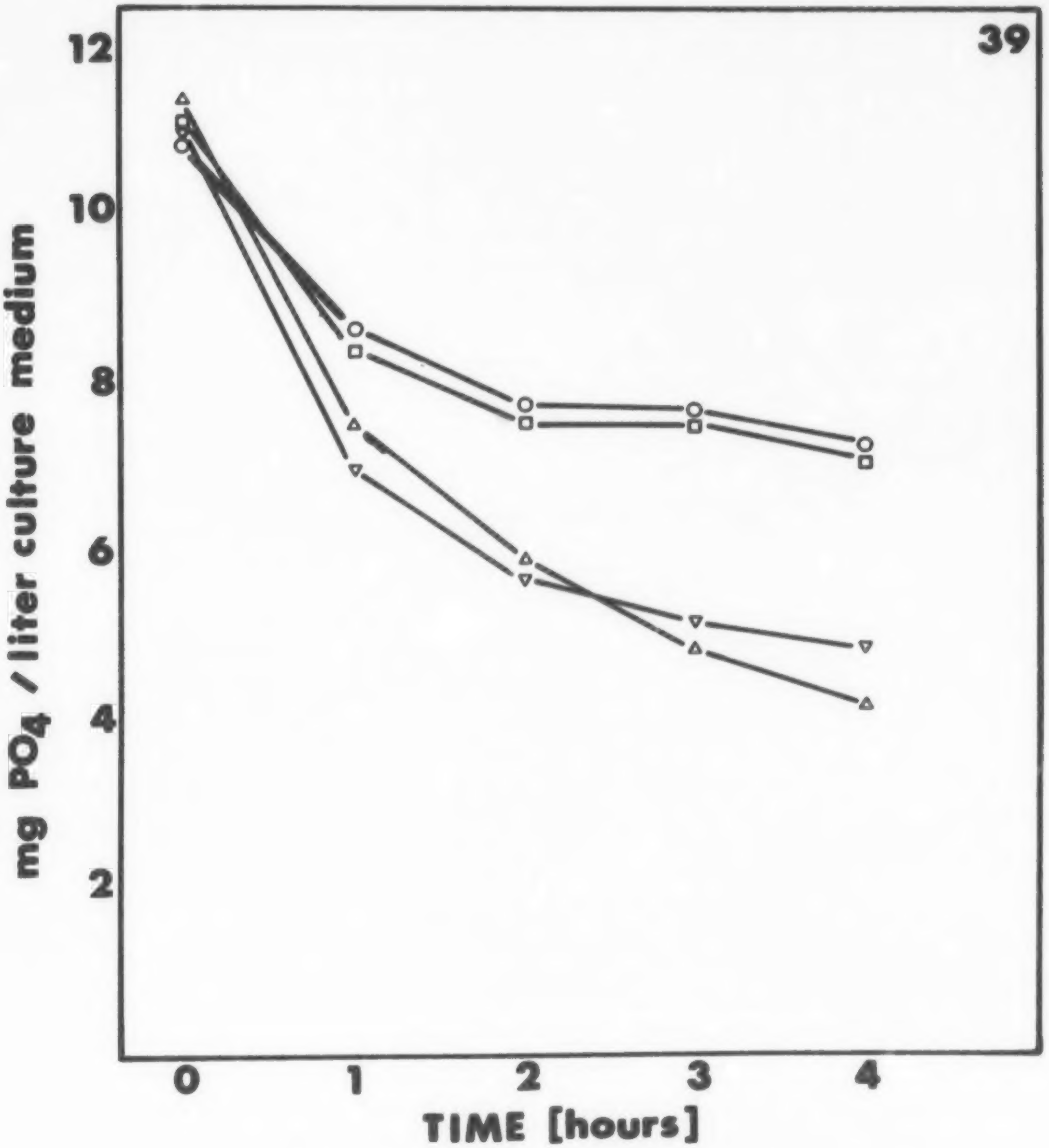
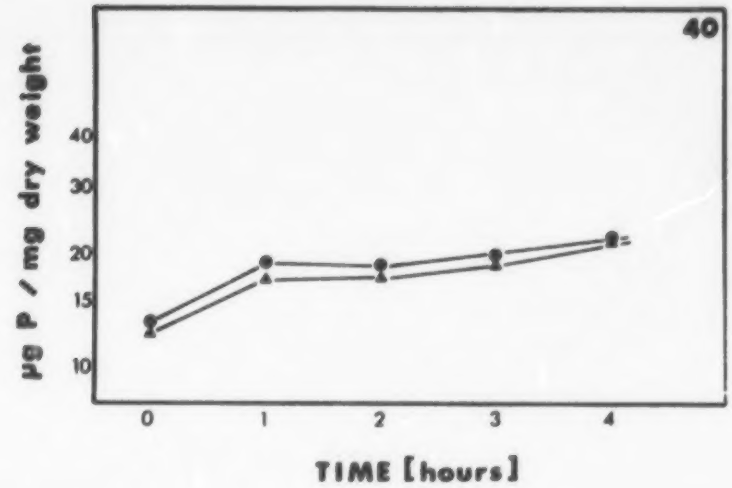


Figure 39. Phosphorus left in medium during rapid uptake by cells starved for 5 days in PO_4 free modified Fitzgerald's medium at a pH of 9. Uptake done at 500 ft-candles, 75°F, pH 9 (adjusted with NaOH) in 300 ml of distilled water containing 10 mg PO_4/l and Na^+ (○), Na^+ and Ca^{++} (△), Na^+ and Mg^{++} (□), and Na^+, Ca^{++} and Mg^{++} (▽). Ions added in same concentration as in Fitzgerald's modified medium except Na^+ was kept at the same level as K^+.



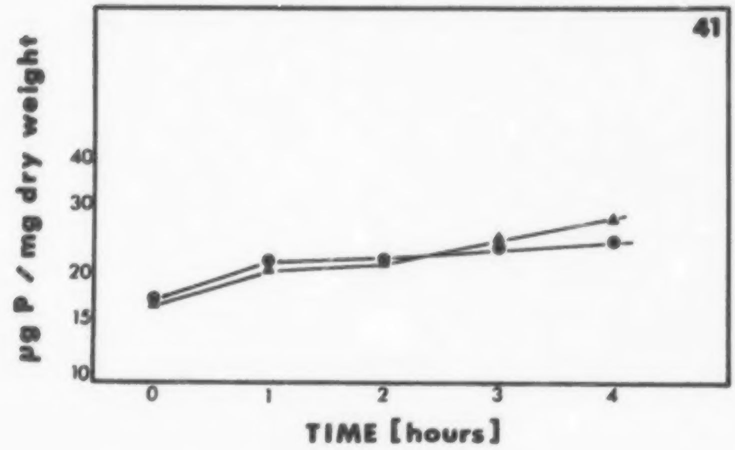


Figure 40. Total cell phosphorus in cells during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO_4 and K^+. Total cell phosphorus during rapid uptake from complete medium (▲) and total cell phosphorus during rapid uptake from medium lacking K^+ (●). $NaH_2PO_4.H_2O$ was used as the phosphorus source. Semi-log plot.

Figure 41. Total cell phosphorus in cells during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO_4 and Ca^{++}. Total cell phosphorus during rapid uptake from complete medium (▲) and total cell phosphorus during rapid uptake from medium lacking Ca^{++} (●). Semi-log plot.

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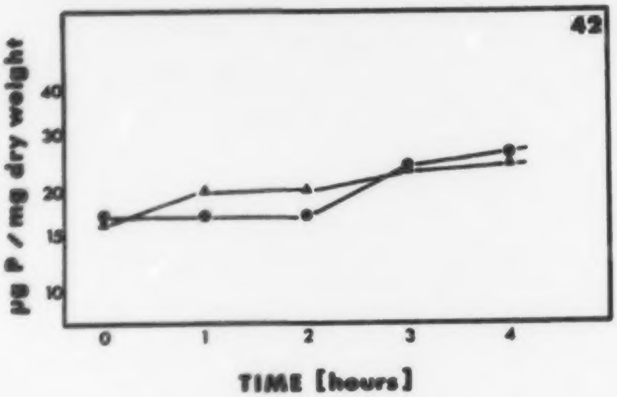

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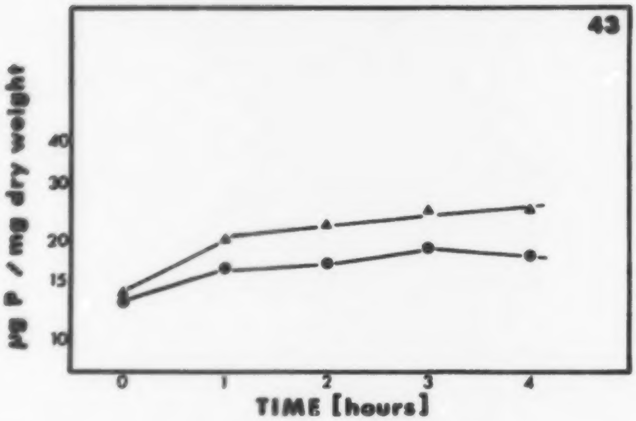
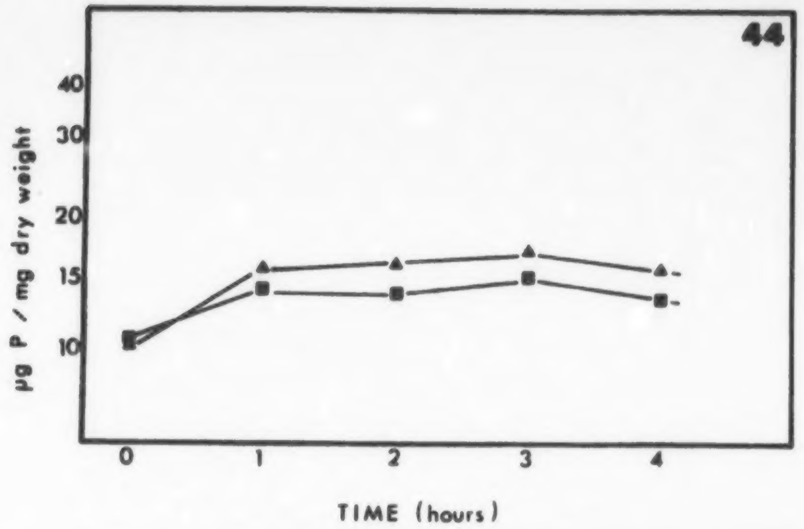


Figure 42. Comparison of total cell phosphorus during rapid uptake of phosphorus after 5 days of starvation in medium without phosphorus and $MgSO_4$. Total cell phosphorus during rapid uptake from complete medium (▲). Total cell phosphorus during rapid uptake from medium without $MgSO_4$ (●). Semi-log plot.

Figure 43. Total phosphorus in cells during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO_4, K^+ and Ca^{++}. Total cell phosphorus during rapid uptake from complete medium (▲) and total cell phosphorus during rapid uptake from medium lacking K^+ and Ca^{++} (●). $NaH_2PO_4.H_2O$ was used as the phosphorus source. Semi-log plot.



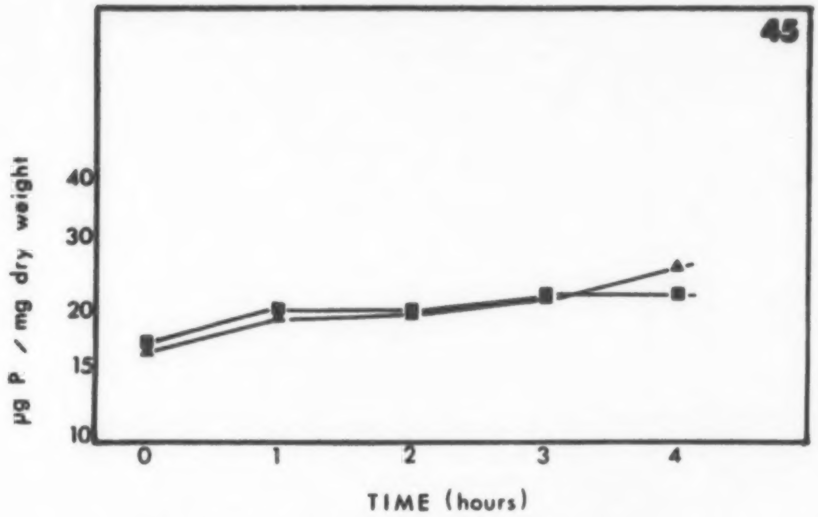
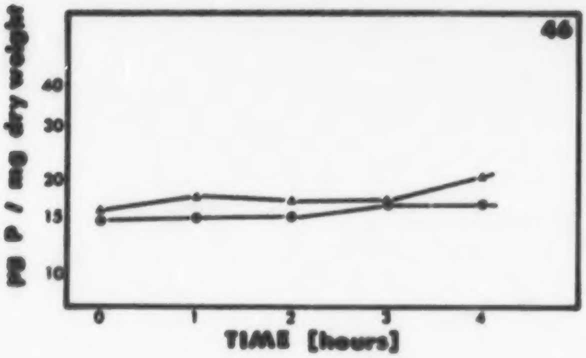


Figure 44. Total cell phosphorus in cells during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO_4, Ca^{++} and Mg^{++}. Total cell phosphorus during rapid uptake from complete medium (▲) and total cell phosphorus during rapid uptake from medium lacking Ca^{++} and Mg^{++} (●). Semi-log plot.

Figure 45. Comparison of total cell phosphorus during rapid uptake of phosphorus after 5 days of starvation in medium without phosphorus and $MgSO_4$. Total cell phosphorus during rapid uptake from complete medium (▲). Total cell phosphorus during rapid uptake from medium without $MgSO_4$ and KH_2PO_4 (●). NaH_2PO_4 was used as the phosphorus source. Semi-log plot.



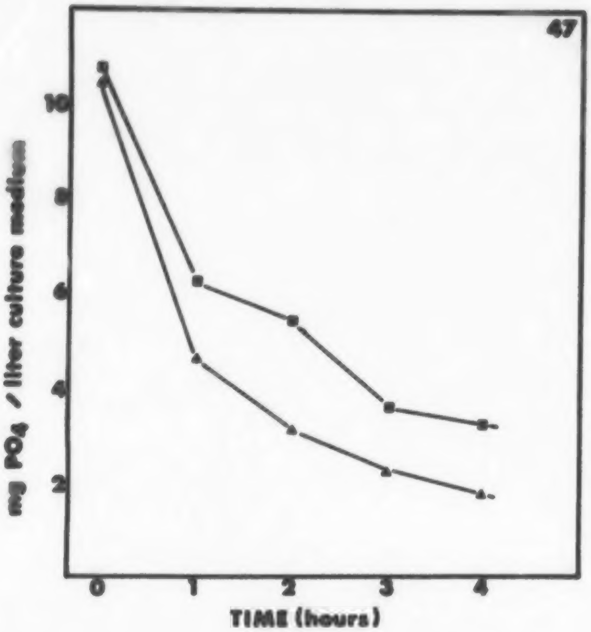


Figure 46. Total phosphorus in cells during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO_4, Mg^{++}, Ca^{++} and K^+. Total cell phosphorus during rapid uptake from complete medium (▲) and total cell phosphorus from medium lacking Mg^{++}, Ca^{++} and K^+ (●). $NaH_2PO_4.H_2O$ was used as the phosphorus source. Semi-log plot.

Figure 47. Comparison of rapid uptake of phosphorus after 5 days of starvation in medium without phosphorus and K^+. Uptake of phosphorus from complete medium (▲). Uptake of phosphorus from medium without K^+ (■).

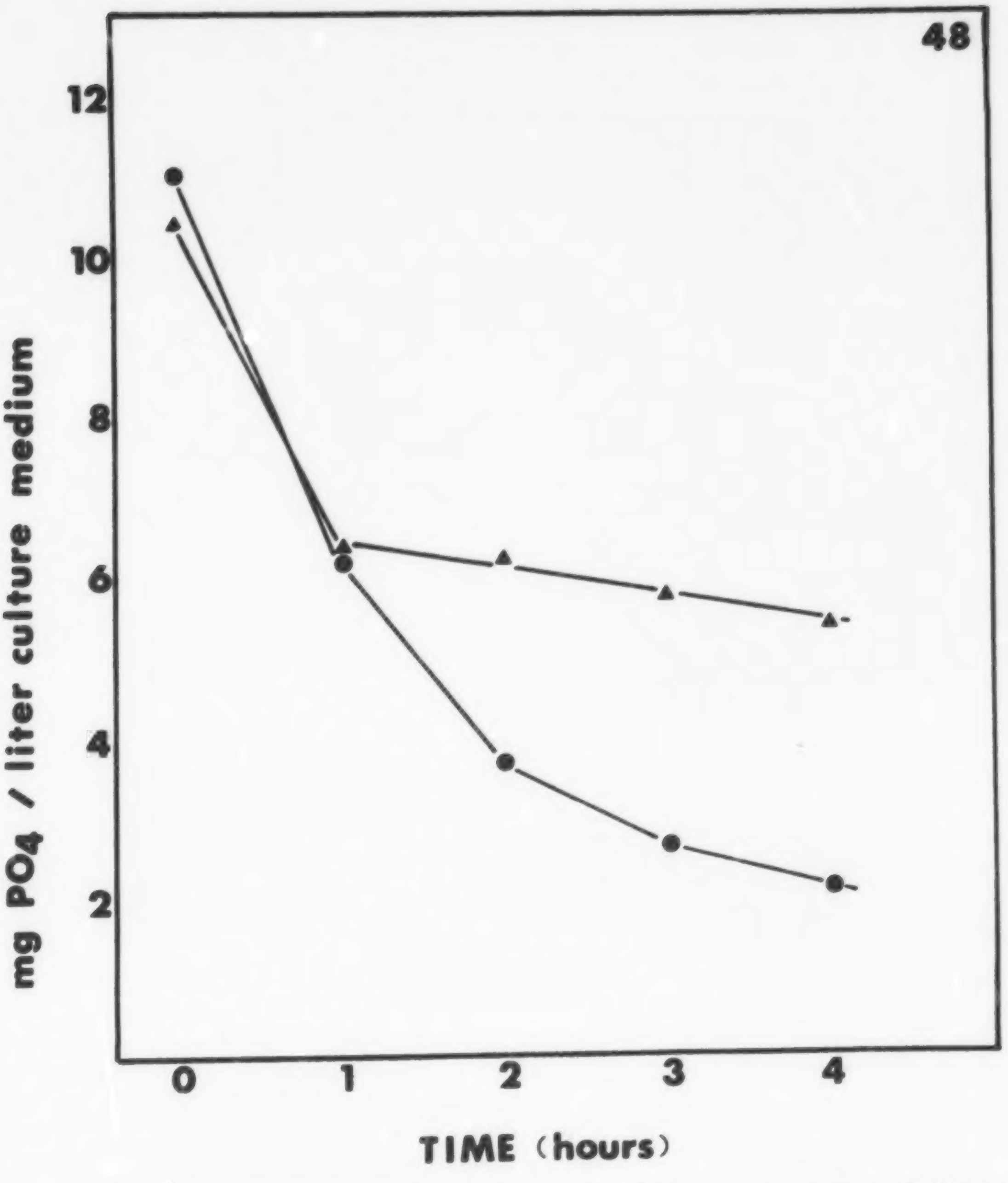


Figure 48. Comparison of rapid uptake of phosphorus after 5 days of starvation in medium without phosphorus, and $CaCl_2$. Uptake of phosphorus from complete medium (▲). Uptake of phosphorus from medium without $CaCl_2$ (●).

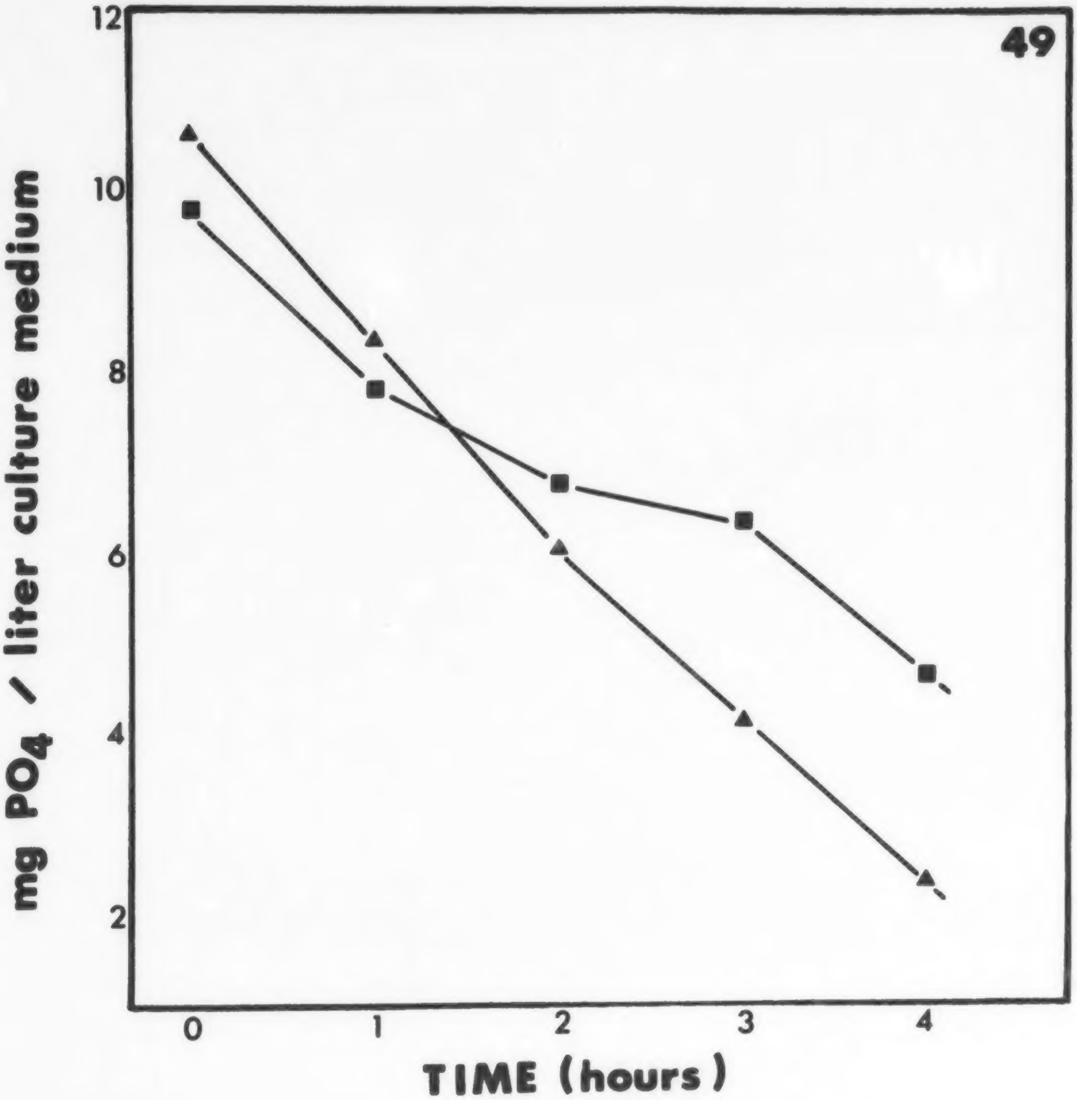
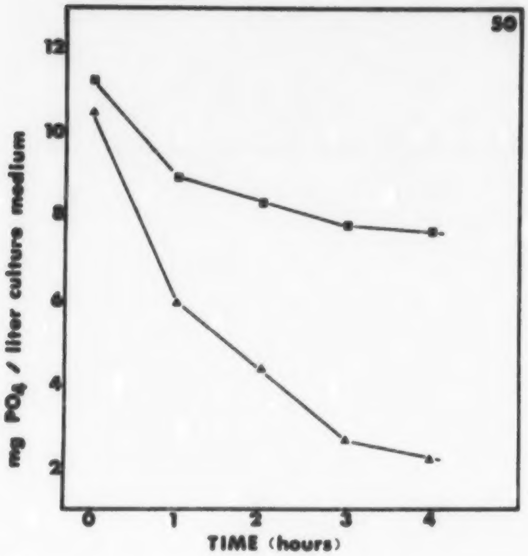


Figure 49. Comparison of rapid uptake of phosphorus after 5 days of starvation in medium without phosphorus and Mg^{++}. Uptake of phosphorus from complete medium (▲). Uptake of phosphorus from medium without $MgSO_4$ (■).



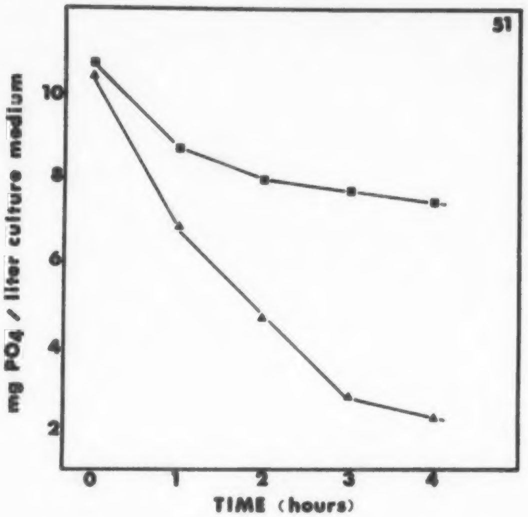
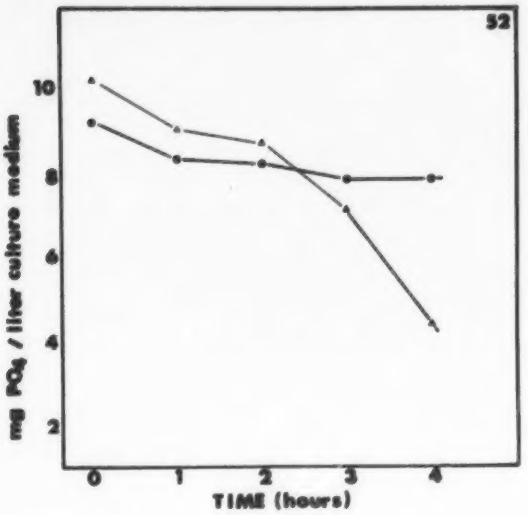


Figure 50. Comparison of rapid uptake of phosphorus after 5 days of starvation in medium without $CaCl_2$ and KH_2PO_4. Uptake of phosphorus from complete medium (▲). Uptake of phosphorus from medium without $CaCl_2$ and K^+ (■). $NaH_2PO_4.H_2O$ was used as the phosphorus source.

Figure 51. Comparison of rapid uptake of phosphorus after 5 days of starvation in medium without phosphorus, $CaCl_2$ and $MgSO_4$. Uptake of phosphorus from complete medium (▲). Uptake of phosphorus from medium without $CaCl_2$ and $MgSO_4$ (■).



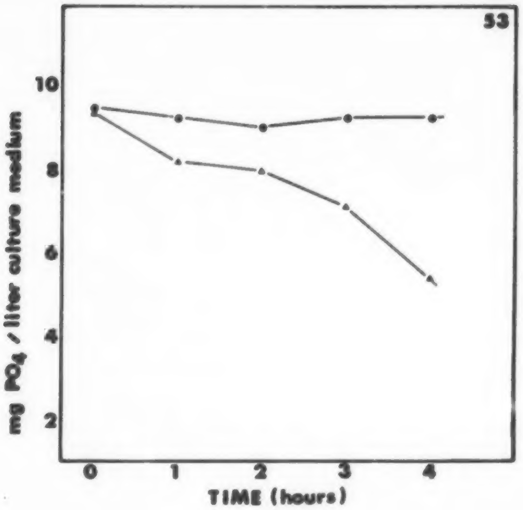


Figure 52. Phosphorus left in the medium during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO$_4$, Mg^{++} and K^{+}. Uptake by cells in complete medium (▲) and uptake by cells in medium without Mg^{++} and K^{+} (●). NaH$_2$PO$_4$.H$_2$O was used as the phosphorus source.

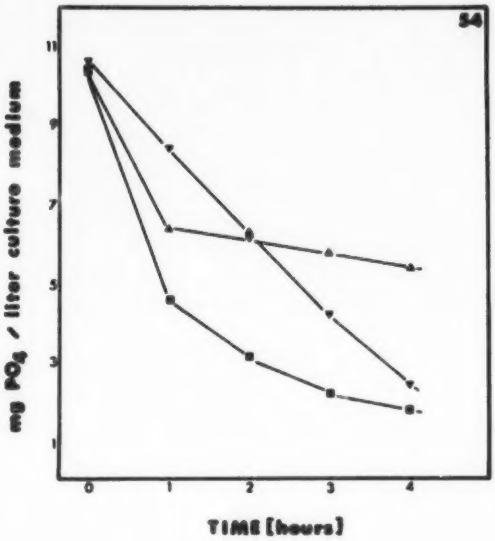
Figure 53. Phosphorus left in the medium during rapid uptake by cells starved for 5 days in modified Fitzgerald's medium without PO$_4$, K^{+}, Ca^{++} and Mg^{++}. Uptake by cells in complete medium (▲) and uptake by cells in medium without K^{+}, Ca^{++} and Mg^{++} (●). NaH$_2$PO$_4$.H$_2$O was used as the phosphorus source.

TABLE 3. SUMMARY OF PHOSPHATE DEPLETION IN THE MEDIUM AND INCREASE IN TOTAL CELL PHOSPHORUS AS A FUNCTION OF EXTERNAL ORTHOPHOSPHATE DEPLETION

Medium Phosphate (mg./l)	Medium Phosphorus (mg./l)		Dry Weight (mg./l)	Medium Phosphorus Dry Weight (mg.P/mg.dw)	Largest increase in Total Cell Phosphorus	Phosphorus Medium after 4 hours of uptake
	conc.	time				
6.58	2.15 0.09	0 4	20	1.03×10^{-1}	9.36×10^{-2}	8.5×10^{-2}
10.13	3.30 0.33	0 4	80	3.7×10^{-2}	1.19×10^{-2}	3.3×10^{-1}
92.51	30.16 24.65	0	75	7.4×10^{-2}	2.73×10^{-2}	24.65

TABLE 4. COMPOSITION OF CULTURE MEDIUM. MODIFIED FITZGERALD'S MEDIUM CONCENTRATIONS OF COMPONENTS EXPRESSED IN MG/LITER

Components	Concentrations (mg/l)
$NaNO_3$	124
$K_2HPO_4.3H_2O$	13
$MgSO_4.7H_2O$	25
$CaCl_2.2H_2O$	36
Na_2CO_3	20
$Na_2SiO_3.9H_2O$	58
Ferric Citrate	3
Citric Acid	3
Gaffron's minor element solution - 0.04 ml	
Gaffron's Solution	(g/l)
H_3BO_3	3.10
$MnSO_4.4H_2O$	2.23
$ZnSO_4.7H_2O$	0.287
$(NH)_{46}MO_7C_{24}.4H_2O$	0.088
$CuSO_4.5H_2O$	0.125
$Co(NO_3)_2.6H_2O$	0.146
$Al_2(SO_4)_3K_2SO_4.24H_2O$	0.474
$NiSO_4(NH_4)_2SO_4.6H_2O$	0.198
$Cd(NO_3)_2.4H_2O$	0.154
$Cr(NO_3)_3.7H_2O$	0.037
$V_2O_4(SO_4)_3.16H_2O$	0.035
$Na_2WO_4.2H_2O$	0.033
KBr	0.119
KI	0.083



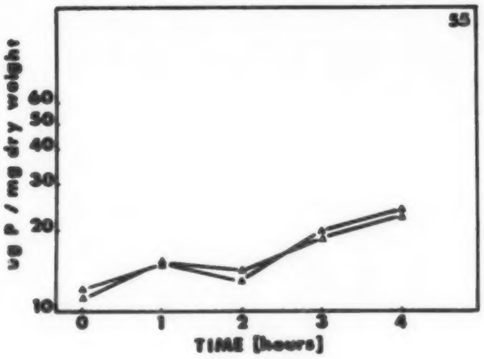
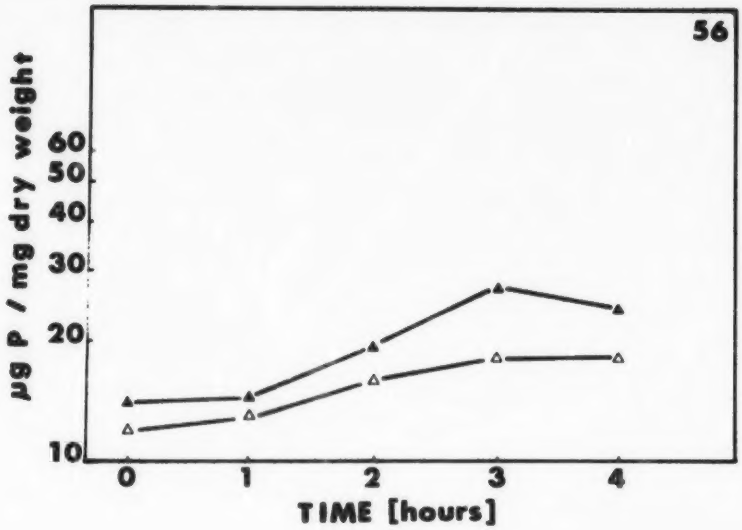


Figure 54. Comparison of phosphorus left in the medium during rapid uptake by cells after 5 days starvation of phosphorus and K^+ (■) phosphorus and Ca^{++} (▲), and phosphorus and Mg^{++} (▼). Uptake was from complete medium.

Figure 55. Total P in cell digest during a four hour rapid uptake of 10 mg PO_4/1 from MFN (△) and MFN with 5 x Mg^{+2} concentration (▲). Cells were starved of P in medium lacking PO_4, but with 5 x Mg^{+2} concentration.



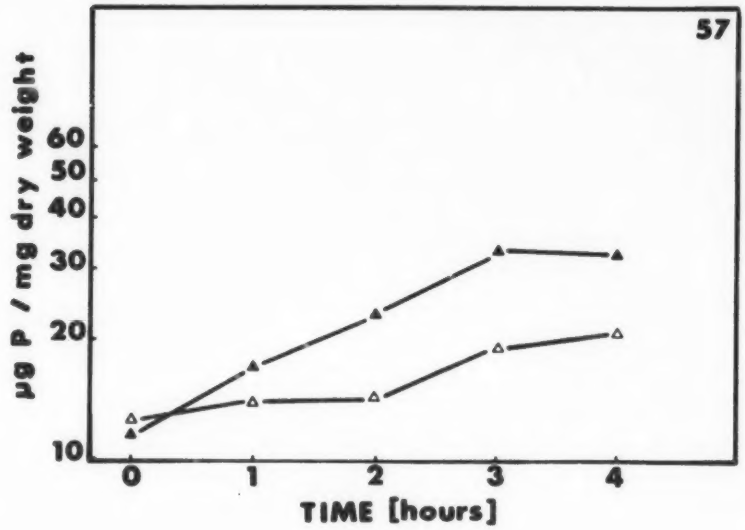


Figure 56. Total P in cell digest during a four hour rapid uptake of 10 mg PO_4/l from MFN (△) and MFN with 5 x K^+ concentration (▲). Cells were starved of P in medium lacking PO_4 but with 5 x K^+ concentration.

Figure 57. Total P in cell digest during a four hour rapid uptake of 10 mg PO_4/l from MFN (△) and MFN with 5 x Ca^{+2} concentration (▲). Cells were starved of P in medium lacking PO_4 but with 5 x Ca^{+2} concentration.

greater than 1 percent transmittance, in three different extractions are summarized in this table. Residues from each successive extraction were also tested as a check on the extraction procedure.

It can be seen that the phosphate levels for the total cell digest, cold TCA extraction, and hot TCA extraction increase with increasing external orthophosphate concentration. This effect is not seen with the lipid extractions or residue. The phosphorus levels in these fractions were highest when the algae were grown in 10 mg PO_4 per liter. Since the algae were normally cultured in medium containing between 8 and 10 mg PO_4 per liter, the values listed for these concentrations in Table 5 were used as the normal phosphorus levels in these fractions.

PHOSPHORUS DISTRIBUTIONS DURING STARVATION AND RAPID UPTAKE

Table 6 summarizes the changes in phosphorus content of the various extracts during five days of starvation and four hours of rapid uptake. These results are also presented in Figures 58-62. It was not possible chemically to extract and analyze hourly all fractions during rapid uptake from one sample due to the length of time required for one extraction. The time required for one total extraction is approximately four hours. Consequently, hours of uptake were monitored in all phosphorus containing fractions whereas 1 and 3 hours were monitored for total cell phosphorus.

Total Cell Digest

Figure 58 demonstrates the changes in phosphorus content of the algae during starvation and uptake. The values of these fractions are also presented in numerical form in Table 6. During the 5 day starvation period, the total phosphorus content varied along with the dry weight of the algae. Five days of starvation led to a reduction of the total phosphorus content of the algae by approximately one-half. Upon the addition of phosphate, this value increased by an order of magnitude of one hour and shows an additional small increase at four hours.

Cold TCA Extractable Phosphorus

The values for this fraction follow a similar pattern for the starvation period (Figure 59 and Table 6). Again, the phosphorus content of the cold TCA extract was reduced by about one-half at the end of the 5 day starvation period. However, the most substantial increase was found at four hours of uptake, when the value was one order of magnitude higher than that of the value for 5 days of variation.

Lipid Extraction

The phosphorus content of the lipid fraction was reduced at the end of the five day starvation period by 81 percent (Figure 60 and Table 6). At the end of the four hour uptake period, this value did not return to the levels found in normal cells, indicating that the phosphate lost during starvation was not recovered in this fraction within four hours.

TABLE 5. PHOSPHORUS CONTENT OF CELL FRACTIONS AS A FUNCTION OF A PHOSPHATE CONCENTRATION IN THE CULTURE MEDIUM. VALUES REPORTED ARE mg P/mg DRY WEIGHT

Phosphate in Medium	Phosphorus Content of Cell Fractions						
	TOTAL CELL DIGEST	COLD TCA EXTRACTION	RESIDUE FROM COLD TCA	LIPID EXTRACTION	LIPID FREE RESIDUE	HOT TCA EXTRACTION	RESIDUE
1 mg.	3.01×10^{-3} $\pm 0.38 \times 10^{-3}$	1.72×10^{-3} $\pm 0.36 \times 10^{-3}$	1.97×10^{-3} $\pm 0.32 \times 10^{-3}$	1.20×10^{-4} $\pm 0.33 \times 10^{-4}$	1.44×10^{-3} $\pm 0.25 \times 10^{-3}$	1.56×10^{-3} $\pm 0.18 \times 10^{-3}$	1.13×10^{-3}
10 mg.	8.25×10^{-3} $\pm 3.0 \times 10^{-3}$	5.51×10^{-3} $\pm 2.33 \times 10^{-3}$	5.85×10^{-3} $\pm 1.40 \times 10^{-3}$	7.54×10^{-4} $\pm 1.67 \times 10^{-4}$	6.71×10^{-3} $\pm 2.16 \times 10^{-3}$	4.49×10^{-3} $\pm 2.08 \times 10^{-3}$	3.23×10^{-3} $\pm 1.07 \times 10^{-3}$
100 mg.	3.28×10^{-2} $\pm 1.0 \times 10^{-2}$	2.79×10^{-2} $\pm 2.31 \times 10^{-2}$	7.58×10^{-3} $\pm 1.31 \times 10^{-3}$	3.37×10^{-4}	6.54×10^{-3} $\pm 2.06 \times 10^{-3}$	5.67×10^{-3} $\pm 0.72 \times 10^{-3}$	1.89×10^{-3} $\pm 1.34 \times 10^{-3}$
1000 mg.	3.58×10^{-2} $\pm 1.46 \times 10^{-2}$	2.00×10^{-2} $\pm 1.85 \times 10^{-2}$	4.80×10^{-3} $\pm 3.08 \times 10^{-3}$	2.30×10^{-4}	2.53×10^{-3}	1.95×10^{-3}	1.27×10^{-3}

TABLE 6. PHOSPHORUS CONTENT OF CELL FRACTIONS DURING 5 DAYS OF PHOSPHATE STARVATION AND 4 HOURS OF PHOSPHATE UPTAKE. SAMPLE DRY WEIGHTS FOR STARVED CELLS ARE PRESENTED TO DEMONSTRATE THE FLUCTUATIONS IN DRY WEIGHT THAT OCCUR DURING STARVATION. THE INCREASE IN DRY WEIGHT IN A REPRESENTATIVE UPTAKE CAN BE SEEN IN FIGURE 21. VALUES REPORTED ARE mg P/mg DRY WEIGHT

growth conditions	TOTAL PHOSPHORUS IN CELL FRACTIONS							
	Dry Wt.	Total Cell Digest	Cold TCA	Cold TCA Residue	Lipid Extract	Lipid Free Residue	Hot TCA Extract	Residue
Normal Cells 8-10mg.		8.25×10^{-3} $\pm 3.00 \times 10^{-3}$	5.51×10^{-3} $\pm 2.33 \times 10^{-3}$	5.85×10^{-3} $\pm 1.40 \times 10^{-3}$	7.54×10^{-4} $\pm 1.18 \times 10^{-4}$	6.71×10^{-3} $\pm 2.16 \times 10^{-3}$	4.49×10^{-3} $\pm 2.08 \times 10^{-3}$	3.23×10^{-3} $\pm 1.07 \times 10^{-3}$
1 Day Starved	1200	5.35×10^{-3}	2.52×10^{-3}		1.09×10^{-4}		2.52×10^{-3}	1.75×10^{-3}
2 Day Starved	2400	2.48×10^{-3}	1.17×10^{-3}		7.0×10^{-5}		1.29×10^{-3}	8.7×10^{-4}
3 Day Starved	1400	5.38×10^{-3}	1.97×10^{-3}		9.3×10^{-4}		1.91×10^{-3}	3.12×10^{-3}
4 Day Starved	2000	2.98×10^{-3}	1.27×10^{-3}		1.4×10^{-4}		1.21×10^{-3}	1.72×10^{-3}
5 Day Starved		4.19×10^{-3} $\pm 0.92 \times 10^{-3}$	2.35×10^{-3} $\pm 1.08 \times 10^{-3}$	5.73×10^{-3}	1.03×10^{-4}	5.1×10^{-3}	2.68×10^{-3} $\pm 1.56 \times 10^{-3}$	3.3×10^{-3} $\pm 2.12 \times 10^{-3}$
1 Hour Uptake		4.43×10^{-2} $\pm 1.86 \times 10^{-2}$	7.02×10^{-3}					
2 Hour Uptake		4.78×10^{-2} $\pm 1.96 \times 10^{-2}$	7.70×10^{-3}		3.5×10^{-3}			8.6×10^{-3}
3 Hour Uptake		4.69×10^{-2} $\pm 1.75 \times 10^{-2}$	7.20×10^{-3}					
4 Hour Uptake		7.83×10^{-2} $\pm 1.52 \times 10^{-2}$	2.35×10^{-2} $\pm 0.77 \times 10^{-2}$	4.40×10^{-3}	5.6×10^{-3}		3.88×10^{-2}	9.0×10^{-3}

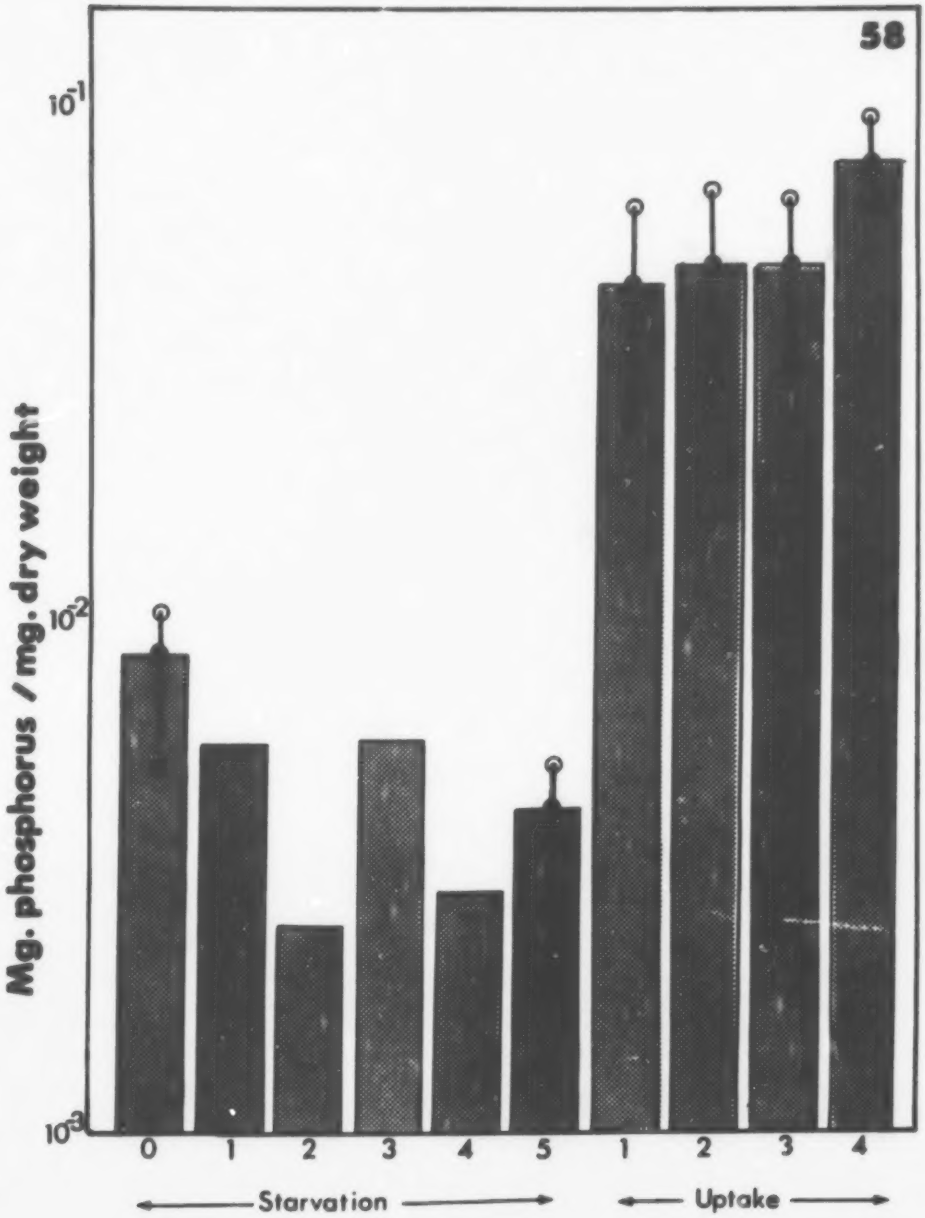


Figure 58. Semi-logarithmic graphic presentation of the change in Total Cell Phosphorus during starvation and rapid uptake. The standard error of the samples is presented in this figure and in all subsequent figures as open circles connected by lines. Standard errors are plotted where four or more samples were analyzed. All other data represents the mean of three extractions.

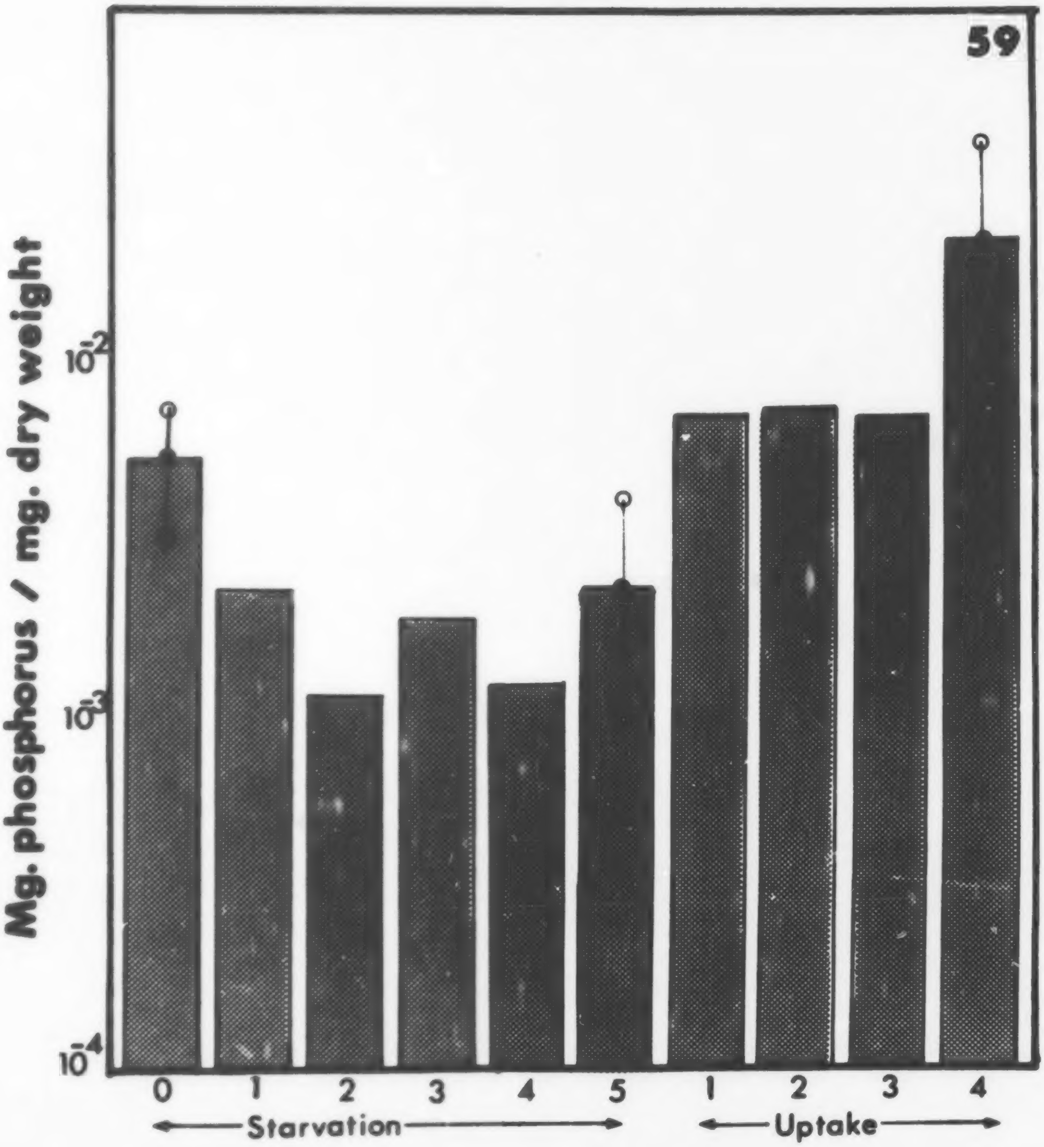


Figure 59. Semi-logarithmic graphic presentation of the change in cold TCA extractable phosphorus during starvation and rapid uptake.

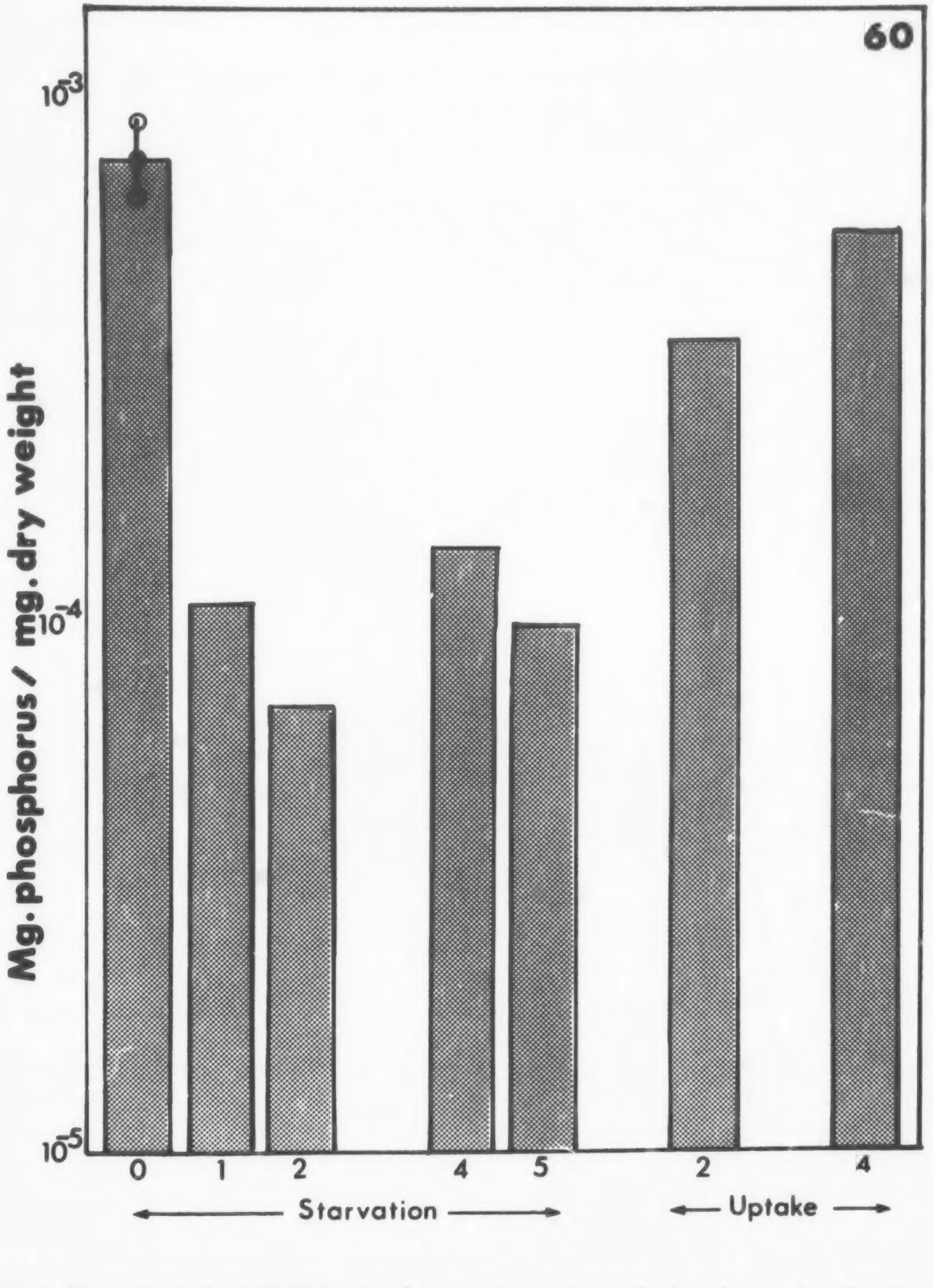


Figure 60. Semi-logarithmic graphic presentation of the change in phosphorus content of lipid fraction during starvation and rapid uptake.

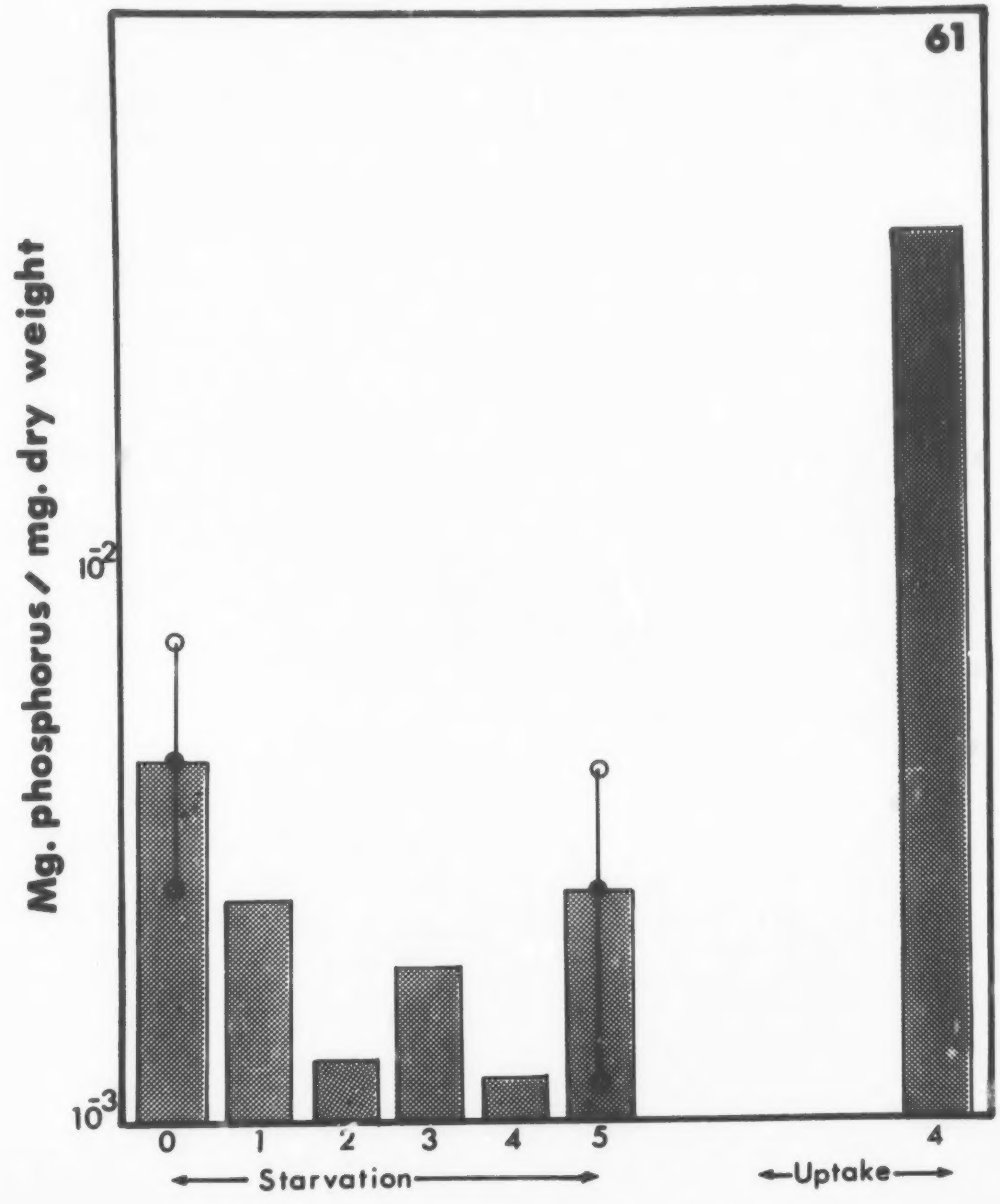


Figure 61. Semi-logarithmic presentation of the change in hot TCA extractable phosphorus during starvation and rapid uptake. Hourly sampling of the hot TCA extract was difficult, and the results obtained were questionable. Therefore, only the sample taken on the fourth hour was plotted.

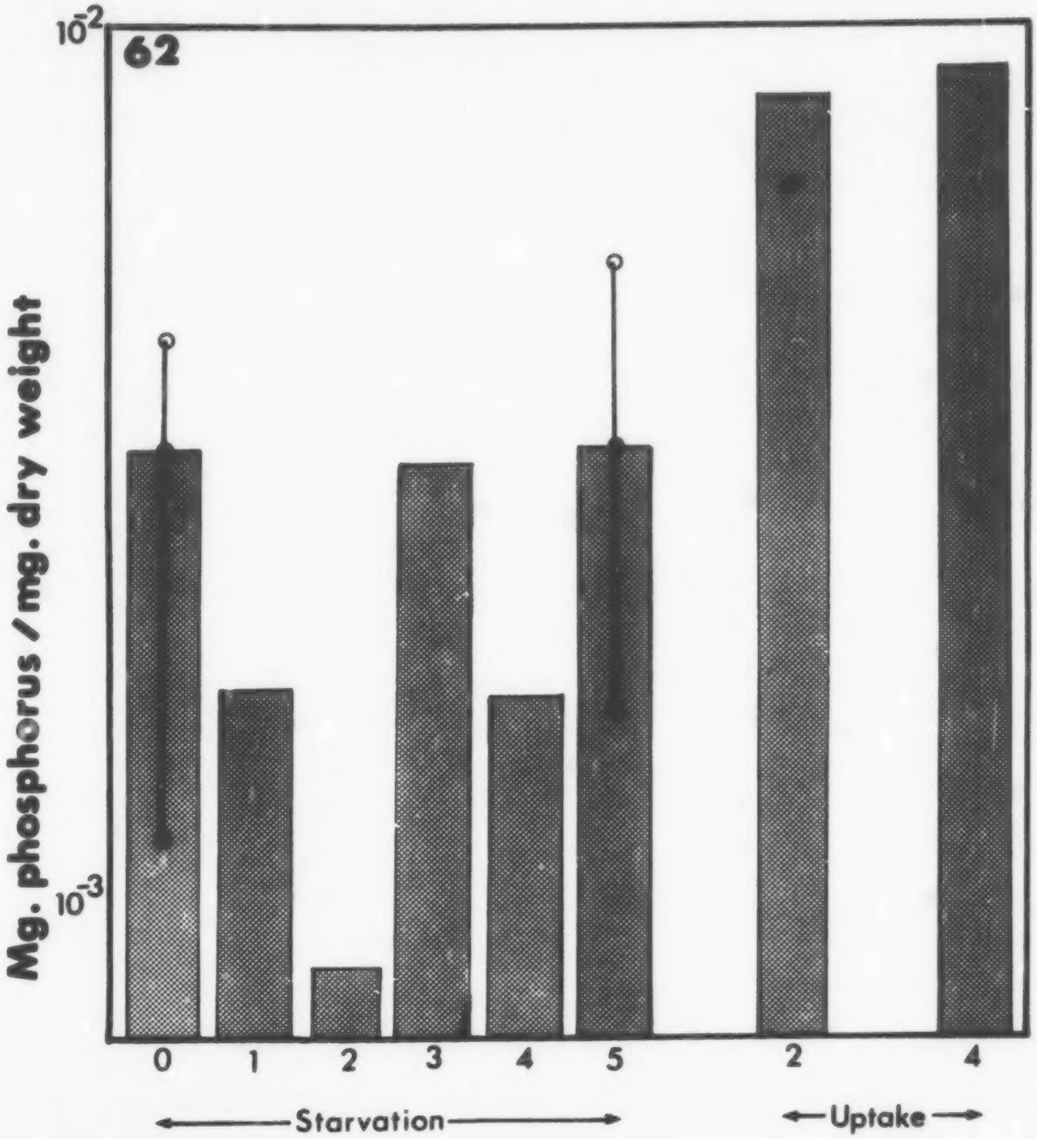


Figure 62. Semi-logarithmic graphic presentation of the change in phosphorus content of the residue from the threefold extraction during starvation and rapid uptake.

Hot TCA Extractable Phosphorus

The greatest increase in phosphorus content of any cell fraction during the uptake period was found in the hot TCA extract (Figure 61 and Table 6). Five days of starvation led to a reduction in this fraction by approximately 40 percent. At the end of the four hour uptake period, the increase in phosphorus content was greater than an order of magnitude, the value increasing from 2.68 to 38.8 µg P per mg dry weight algae.

It is also interesting to note that although in normal cells the amount of phosphorus present in the hot TCA extract was less than that in the cold extract, the values at four hours of uptake were reversed. There was more phosphorus present in the hot TCA extract. During the five day starvation period, these values were approximately the same.

Residue From The Three-Fold Extraction

The residue from the three-fold extraction as previously described showed the greatest variation in phosphorus content during the starvation period (Figure 62 and Table 6). However, the phosphorus content of this fraction was essentially the same for a 5 day starved culture and a normal culture. The increase after four hours of uptake was also not as large as the other extractions. This fraction probably represents all phosphorus compounds in the cell other than some nucleic acids, short and long chain polyphosphates, orthophosphate, and phospholipids.

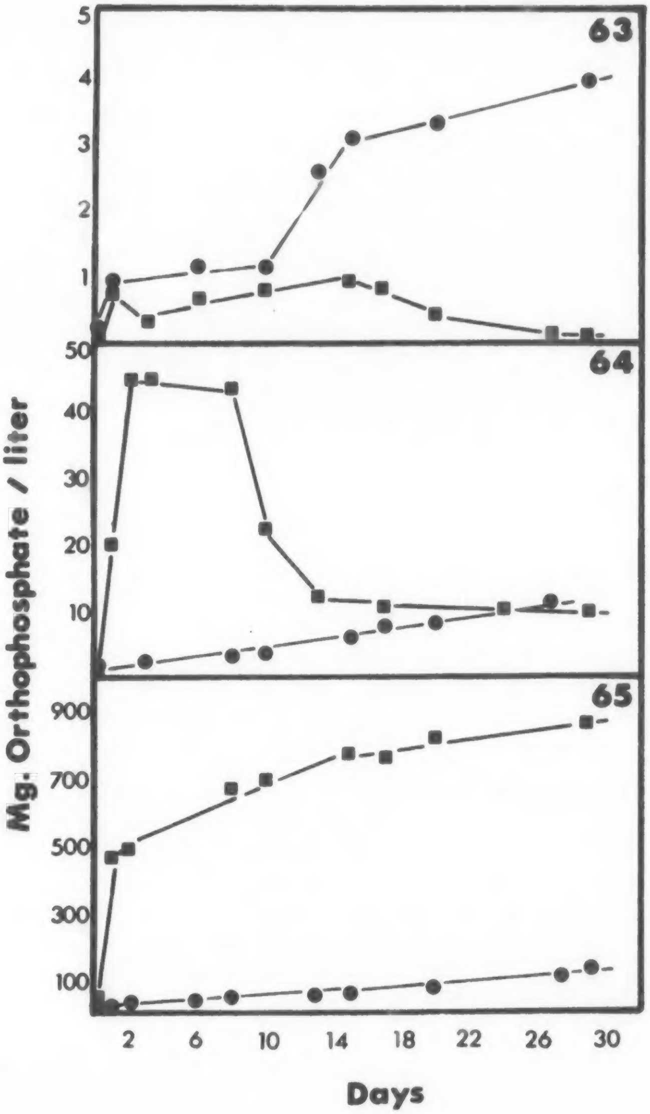
HYDROLYSIS OF CONDENSED PHOSPHATES

Hydrolysis of the condensed phosphate, sodium tripolyphosphate, was tested both with and without the experimental organism. Modified Fitzgerald's medium was made with three different concentrations of the condensed phosphates, 4.59, 153.7 and 1204 mg PO_4 per liter culture medium. The tubes were incubated under normal culture conditions as previously described. These results are presented in Figures 63-65.

Figure 63 demonstrates that at a concentration of 4.59 mg PO_4 per liter, the condensed phosphate apparently hydrolyzes at a greater rate with the alga present in the medium. At a concentration of 153.7 mg $PO_4/1$ (Figure 64), the condensed phosphate hydrolyzes more rapidly in the presence of the alga. However, this value begins to decrease on the tenth day of culture, and does not rise again. Hydrolysis in all cases is determined by an increase in orthophosphate concentration in the medium. Figure 65 illustrates the rapid hydrolysis of a considerable amount of condensed phosphate on the first day of culture, and a subsequent steady increase in the amount of orthophosphate present in medium. At the higher concentrations of condensed phosphate (Figures 64 and 65), hydrolysis in the absence of algae proceeded at a constant rate.

RAPID UPTAKE OF CONDENSED PHOSPHATES

Sodium tripolyphosphate was substituted for orthophosphate in a normal starvation and rapid uptake experiment. Total cell fractionations of phosphorus-containing compounds were assayed. These results are presented in Figure 66.



Figures 63-65. Hydrolysis of sodium tripolyphosphate in Modified Fitzgerald's medium both in the presence and absence of P. boryanum. Circles represent hydrolysis without cells, and squares represent hydrolysis with cells present. Hydrolysis is indicated by an increase in orthophosphate present in the medium.

Figure 63. 4.59 mg PO_4/liter culture medium

Figure 64. 153.7 mg PO_4/liter culture medium

Figure 65. 1204 mg PO_4/liter culture medium

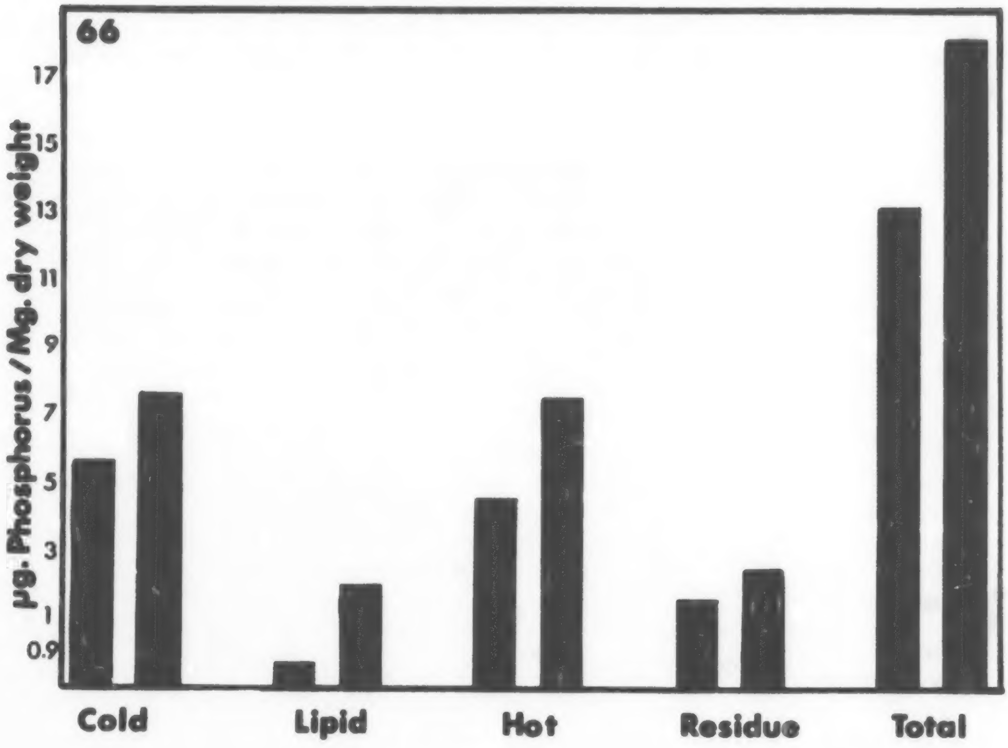


Figure 66. Increase in phosphorus-containing fractions during starvation and rapid uptake from 10 mg PO_4 (as sodium tripolyphosphate) per liter. The left bar of each pair represents starved cells, and the right bar represents 4 hours of uptake. The uptake with condensed phosphate was only repeated twice. Consequently the values reported represent the mean.

There was no substantial increase in any cell fraction after four hours of uptake. This is in contrast to an uptake with orthophosphate as previously discussed. Refer again to Table 6 and Figures 58-62.

CELL ULTRASTRUCTURE UNDER NORMAL CULTURE CONDITIONS

Cells of *P. boryanum* grown under normal culture conditions have an appearance which is similar to that of other blue-green algae. Cellular inclusions such as polyhedral bodies, areas of DNA, ribosomes, thylakoids, lipid droplets, cyanophycin granules, and a few small polyphosphate bodies are found in the cell with regularity (Figures 67 and 68). A four-layered cell wall, as described in most blue-green algae is also present (Figure 68). Some intrathylakoidal vacuolization is present, with the thylakoids usually arranged at the periphery of the cell (Figure 67). Cell division occurs by transverse binary fission. A newly formed septum can be seen in Figure 67.

CHANGES IN ULTRASTRUCTURE ASSOCIATED WITH SEVERAL PHOSPHATE CONCENTRATIONS

100 mg PO_4 per liter

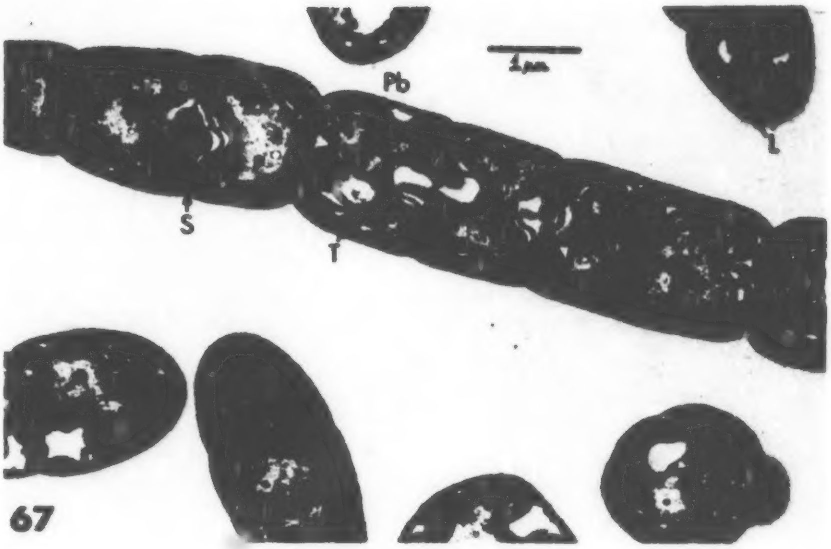
P. boryanum grown in culture medium containing 100 mg PO_4 per liter had a number of alterations in cell structure. Figure 69 demonstrates these changes. Generally, there is a marked increase in cell death and cell lysis, as evidenced by the debris in the sectioned pellet. The cells which appear to be dead have cytoplasm which is devoid of ribosomes. The thylakoids are also fewer in number and are rather prominent in the cell. Occasionally, large polyphosphate bodies, or areas where polyphosphate bodies have fallen out, can be seen. Cells which are normal in appearance, as previously described, can also be found.

1000 mg PO_4 per liter

Changes that occured in culture at 100 mg per liter were even more pronounced when the algae were grown in medium containing 1000 mg PO_4 per liter (Figures 70-72). Figure 73 demonstrates some expansion of thylakoids (IT), and a large hole in the center of the cell, presumably an area where a polyphosphate body has fallen out during sectioning. In the more normal appearing cells, the thylakoids are still located at the periphery, and the DNA is located in the center of the cell. Figure 71 shows in greater detail a cell which appears to be dead, and is devoid of ribosomes. The frequency of encountering such cell types increases with greater phosphate concentrations. Figure 72 shows a portion of a filament containing several polyphosphate bodies which have sublimated under the electron beam or have been partially lost during the section preparation procedures. The ribosomes also have an unusual appearance, being much larger than in cells grown in normal culture medium.

1 mg PO_4 per liter

Cells grown in culture medium containing 1 mg PO_4 per liter have approximately one-tenth of the normal phosphate concentration available to them. This particular culture condition also leads to changes in cell architecture, but these changes are unlike those of higher phosphate concentrations (Figure 73-76). The most pronounced changes that occur are the expansion of intrathylakoidal



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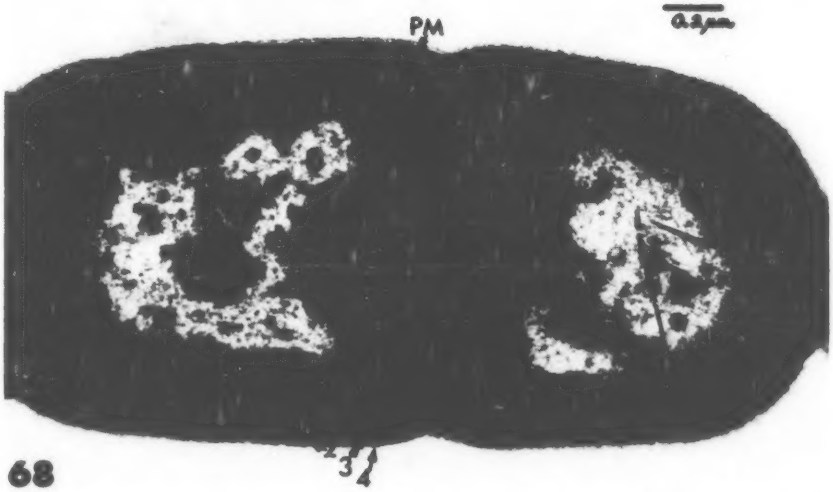

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Figure 67. Cells of P. boryanum grown for 14 days in 10 mg PO_4/liter at 500 ft-candles of illumination with an alternating 12 hour day/night cycle. Note the relatively small intrathylakoidal spaces, and inclusions normally present in blue-green algae such as lipid droplets (L), polyhedral bodies (Pb), and areas of DNA (D). Cell division which will result in two equal daughter cells can also be seen (S).

Figure 68. P. boryanum grown under normal conditions described above and shown at a higher magnification. Note the arrangement of the cell wall outside the plasma membrane (PM). Layer 2 is the mucopolymer-containing layer.

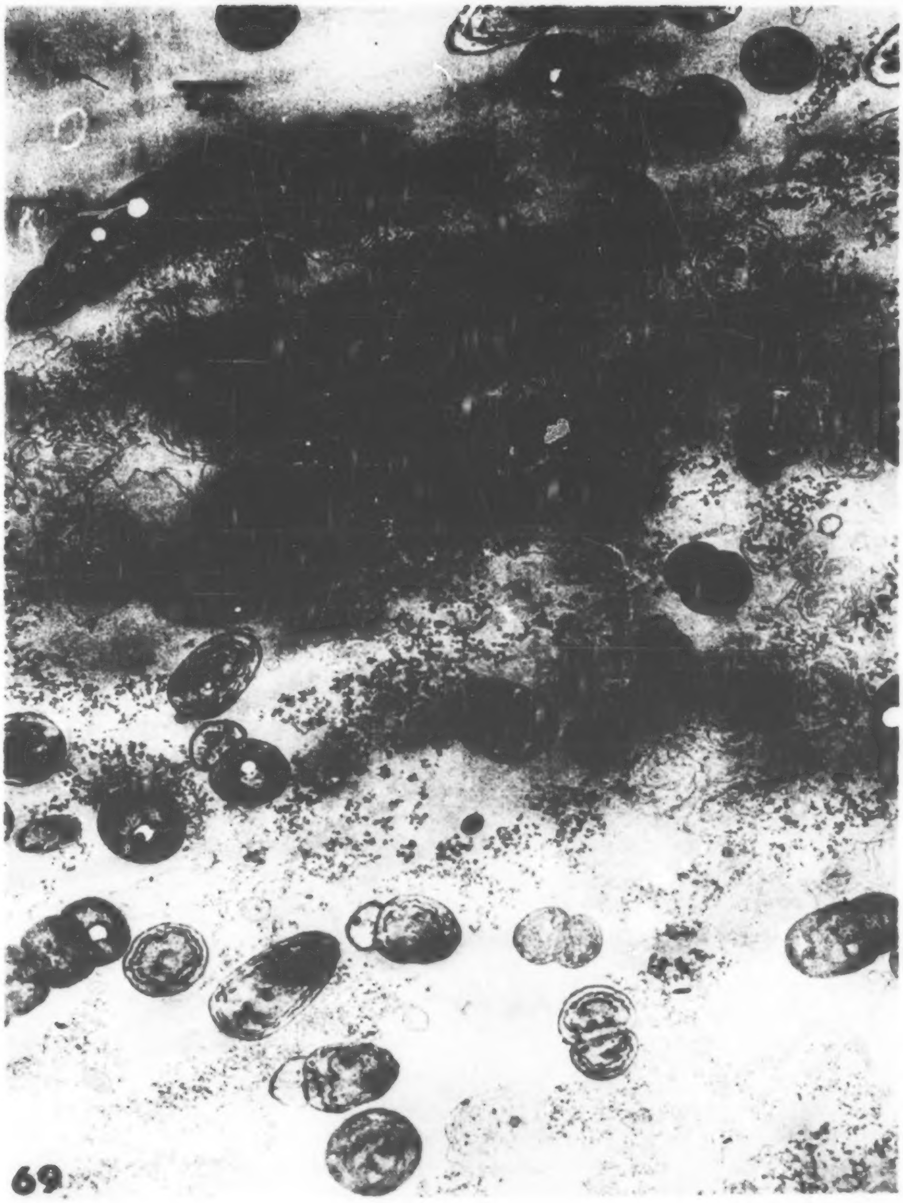

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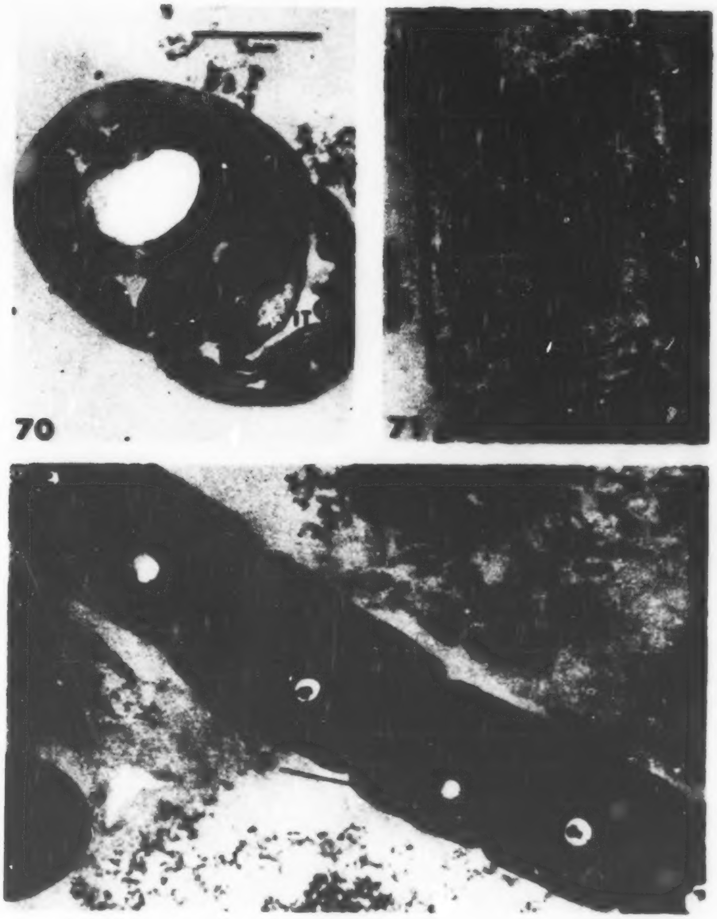
Figure 69. Low power micrograph of a sectioned pellet of P. boryanum grown in Modified Fitzgerald's Medium for 14 days containing 100 mg PO_4/ liter at 500 ft-candles of illumination. Note the cellular debris in the pellet, and the appearnace of several types of cells. The filament in the upper left corner of the picture can be considered as "normal", resembling cells grown under the conditions listed for Figure 38. Other cell types are also evident.

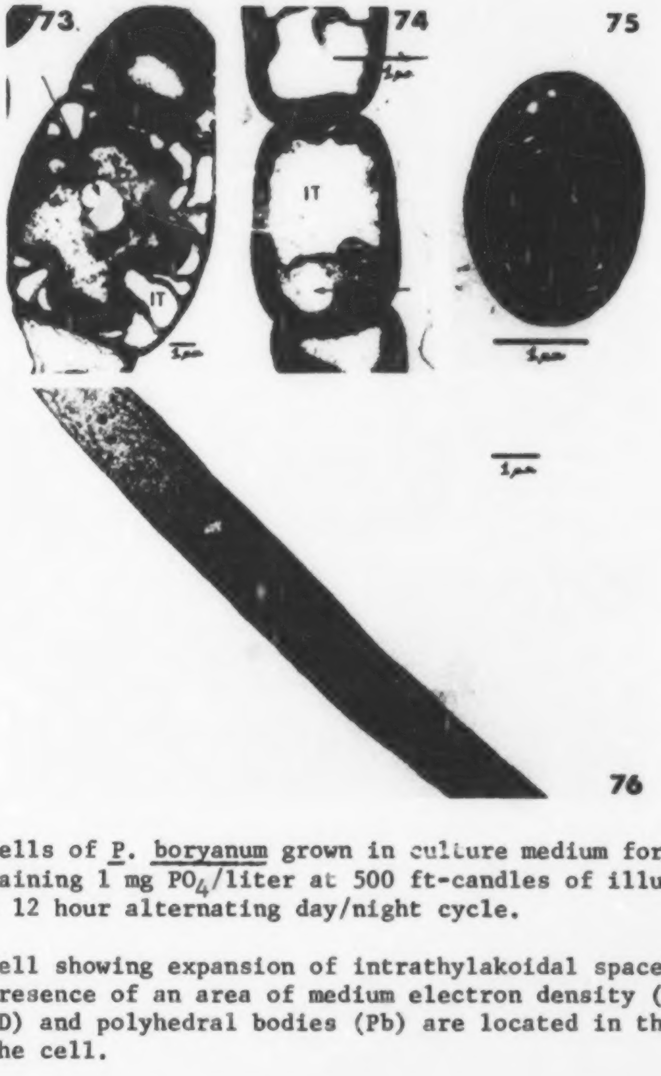


Figures 70-72. Cells of P. boryanum grown for 14 days in medium containing 1000 mg PO_4/liter at 500 ft-candles of illumination. Note the cell types present, as in the culture containing 100 mg PO_4/liter.

Figure 70. Portions of cells showing some expansion of intrathylakoidal spaces (IT), thylakoids (T) at the periphery of the cells, and DNA (D) and polyhedral bodies (Pb) surrounding a space where a polyphosphate body has fallen out.

Figure 71. A cell, apparently dying, which is devoid of ribosomes. Note the small lipid droplets (L) throughout the cell, and the prominent thylakoids (T).

Figure 72. Portion of a filament showing numerous polyphosphate bodies (P) and enlarged ribosomes (R).



Figures 73-76. Cells of *P. boryanum* grown in culture medium for 14 days containing 1 mg PO_4/liter at 500 ft-candles of illumination and a 12 hour alternating day/night cycle.

Figure 73. Cell showing expansion of intrathylakoidal spaces (IT) and the presence of an area of medium electron density (arrow). DNA (D) and polyhedral bodies (Pb) are located in the center of the cell.

Figure 74. Cell showing a greater degree of expansion of intrathylakoidal spaces (IT). An immature polyphosphate body (arrow) can also be seen.

Figure 75. A second cell type found in phosphate-limited cultures. These cells are characterized by unusually large ribosomes (R).

Figure 76. Portion of an elongate cell of *P. boryanum*. The cell contains many small lipid droplets (L) and ribosomes (R).

spaces (IT) and the development of areas of medium electron density (arrows in Figures 73 and 74). In these figures, the appearance of the cytoplasm and some of the ribosomes is normal in all other respects. Some ribosomes (Figure 76) are unusually large. Polyhedral bodies and areas of DNA are also present. One also encounters, with great frequency, elongate cells (Figure 76). These cells often attain lengths of 6-9 µm, whereas a normal cell is about 3 µm. They frequently have abnormally large ribosomes.

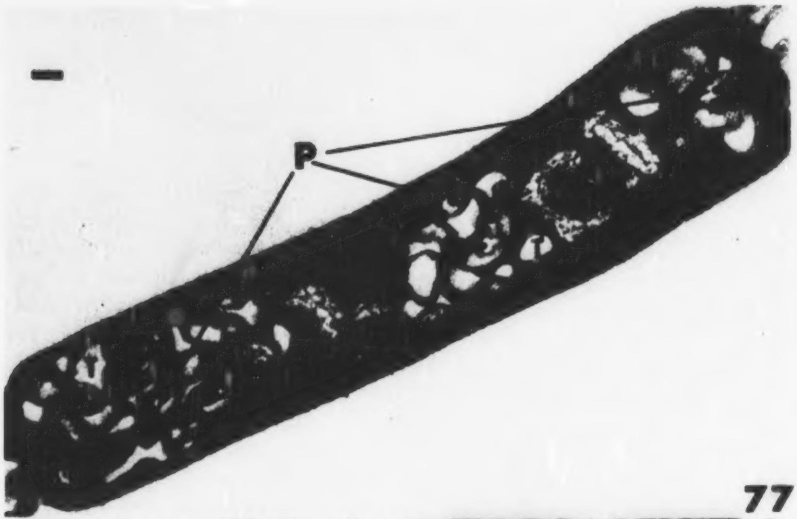
PHOSPHATE-STARVED CELLS

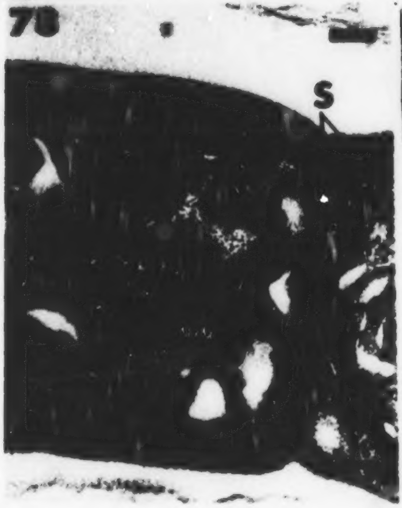
In some cells the cell division process is abnormal. Some cells are abnormally long (Figure 77). In normal cultures the cells are about 1.5 by 3.5 µm. The longest cells observed under phosphate-starved conditions are about 1.5 µm by 10 µm. Generally an entire filament is composed of oblong cells. In other filaments the forming septum, rather than being located in the middle of the cell, is displaced toward one end (Figure 78) resulting in a very short cell (Figure 79). Some of the short cells have a length of only about 10 nm and contain only β granules, ribosomes, thylakoids, and some DNA (Figure 79). In other cells the septa are displaced at points around the filament. If the displacement is quite significant, i.e., the septa are not opposite (unpublished results), a bend occurs in the filament when the septa join. All of these conditions are observed with greater frequency as the length of time in the phosphate-free medium increases.

In certain cells numerous polyphosphate bodies can be seen in association with the DNA fibrils (Figures 80, 81). They vary in size from about 20 nm to 200 nm. Three morphological varieties of polyphosphate bodies can be observed. These are (a) bodies which are porous in appearance, (b) bodies having a dense exterior with an electron-transparent central area, and (c) bodies which are completely electron-dense (Figures 80, 81). A few polyphosphate bodies are found in the ribosomal areas away from nucleoplasmic areas. Several of these polyphosphate bodies have a crystal-like substructure (Figure 83). In other cells an area of medium electron density develops in the nucleoplasmic area (Figure 82). Within this area, which seldom exceeds 0.5 µm in diameter, electron-transparent areas develop (Figure 82). Within the electron-transparent areas polyphosphate bodies are formed. The sequential development appears to be nearly the same as previously reported for the development of polyphosphate bodies in ribosomal areas of _P. boryanum_ (Jensen, 1969). Some polyphosphate bodies also form intrathylakoidally (Figure 77). The frequency of observation of these morphological changes seems to increase with increased time the phosphate-free medium.

In some cells the thylakoids expand greatly to form a large electron-transparent area with a density that resembles the areas found in the nucleoplasmic area (Figure 87).

Electron-dense inclusions, sometimes with a mottled structure, appear in many of the cells as phosphate starvation proceeds (Figures 80, 84, 86). These spherical inclusions are between 0.2 µm and 0.4 µm in diameter and sometimes appear to be limited by an osmiophilic layer about 30 A in thickness (Figure 86).





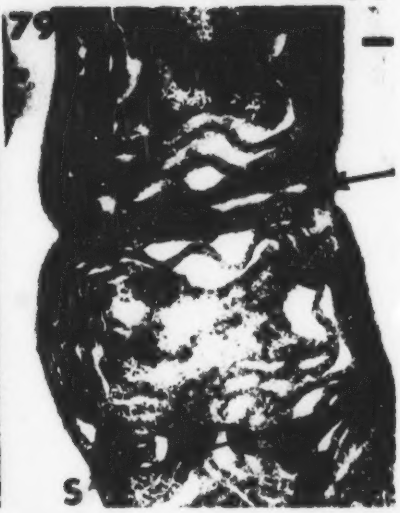
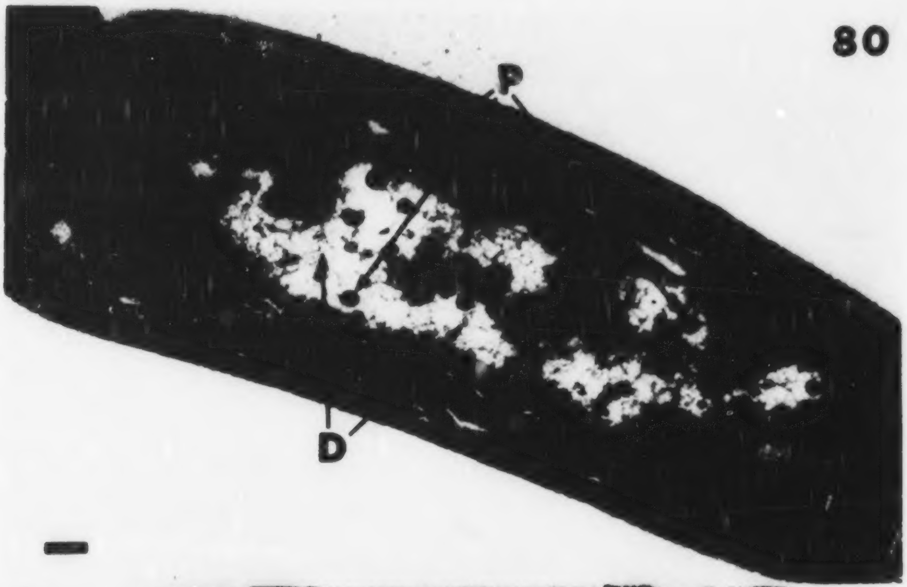


Figure 77. Elongate cell from phosphate-starved culture. Note the expanded thylakoids (T) and small polyphosphate bodies (P). OsO_4 fixation.

Figure 78. Section of portion of a filament from phosphate-starved culture. Note the forming septa (S) which would cut off a small cell. OsO_4 fixation.

Figure 79. Portion of a filament from a phosphate-starved culture. Note the small cell (arrow) which has been formed. Septa (S) are also forming which are not opposite. OsO_4 fixation.

80



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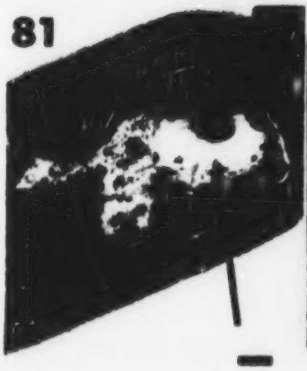

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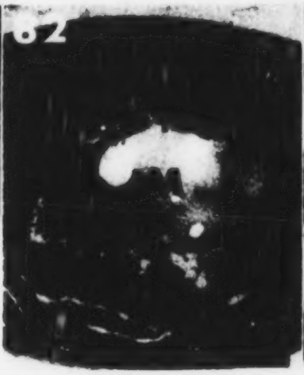


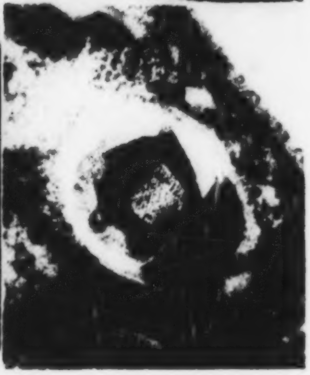
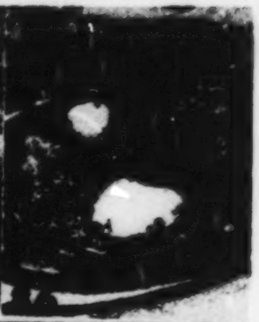


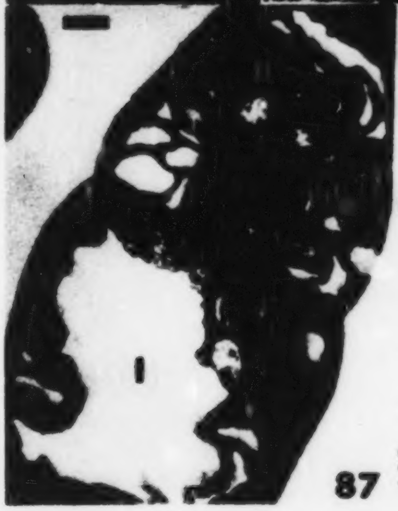
Figure 80. Phosphate-starved cell. Note the large lipid-like inclusions (L) and the numerous polyphosphate bodies (P) associated with DNA fibrils (D). OsO_4 fixation.

Figure 81. Phosphate-starved cell. Note the fibrils (arrow) associated with the polyphosphate body. OsO_4 fixation.

Figure 82. Phosphate-starved cell showing an area of medium electron density (M) with electron-transparent areas. OsO_4 fixation.

Figure 83. Phosphate-starved cell showing a polyphosphate body which has developed in an RNA area. Note the crystal-like organization of part of the body.





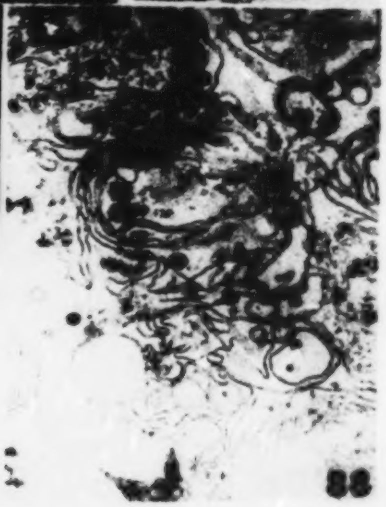


Figure 84. Phosphate-starved cells showing a cyanophycin granule (C) and lipid-like inclusion (L). OsO_4 fixation.

Figure 85. Phosphate-starved cells showing an elongate polyhedral body (Pb). These bodies are always associated with the nucleoplasmic areas (N). OsO_4 fixation.

Figure 86. Phosphate-starved cell with a lipid-like inclusion (L) with a "mottled" appearance which appears to be limited by a 30-A "membrane" (arrow). OsO_4 fixation.

Figure 87. Phosphate-starved cell showing a very large intrathylakoidal space (I) which formed from an expanded thylakoid. OsO_4 fixation.

Figure 88. Micrograph showing the cell debris which is common in phosphate-starved cultures. OsO_4 fixation.

In a number of cells the polyhedral bodies elongage (Figure 85). Generally the polyhedral bodies are about 0.2 µm in diameter. In many starved cells they increase to a length of about 0.6 µm.

Small cyanophycin granules develop during phosphate starvation and generally do not exceed 0.2 µm (Figure 84).

Two types of cells appear in the culture based on the appearance of their ribosomes. Type I has ribosomes which are the usual 20 nm size, and type II has ribosomes which appear abnormally large and measure about 40 nm. Numerous cells fragments are observed in pelleted material (Figure 88), with the 40 nm ribosomes appearing most frequently and suggesting lysis of the type II cells.

RAPID UPTAKE

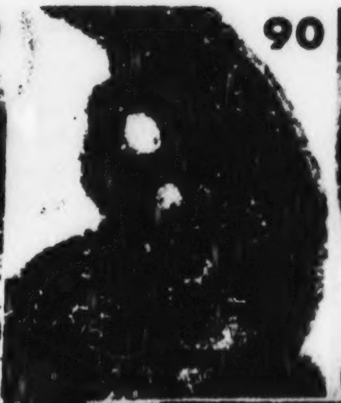
In cells exposed to 10 mg PO_4/liter after 5 days of starvation nearly every cell, after 2 h, contained at least one large polyphosphate body (Figure 92). These bodies are visible at the light-microscope level after staining by the Ebel procedure (Ebel *et al*. 1958). Some cells contain several polyphosphate bodies (Figure 92). Some of these polyphosphate bodies are dense around the periphery while others are completely dense (Figure 92). They vary in diameter from 1.5 µm to the limit of visibility with the light microscope.

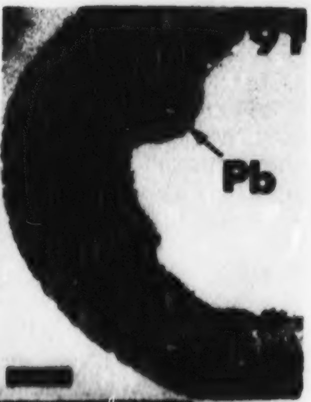
As rapid uptake proceeds many more elongate polyhedral bodies are observed than in starved cells (Figure 89). In many of these polyhedral bodies areas of medium electron density develop in which a smaller electron-transparent area is located (Figures 90, 93). These areas of medium electron density are about 0.05 µm in diameter. Within the electron-transparent areas polyphosphate bodies develop (Figure 91).

The medium electron-transparent areas in the nucleoplasmic area seen occasionally in normal cultures and with increasing frequency in the phosphate-starved cells become very pronounced and most cells contain such areas (Figures 91, 93, 94, 101). Within these areas polyphosphate bodies appear to develop in the electron-transparent areas (Figures 93, 94, 97, 98-101). In some cells only one polyphosphate body is visible in a section through this area (Figure 94) while in other cells several polyphosphate bodies are seen (Figure 97). The diameter of these polyphosphate bodies varies from about 100 nm to 1.5 µm. Associated with most of these polyphosphate bodies are apparent DNA fibrils (Figures 94, 97). These polyphosphate bodies are quite variable in morphology. In addition to the three morphological varieties already described for the starved cells, one can also observe bodies which are irregular in shape (Figures 97, 99, 101) and those which have a compound structure (Figures 98, 99, 100).

In some cells the polyphosphate bodies are small (10-15 nm in diameter) and are associated with the DNA (Figure 95). Their morphology varies as previously described.

In other cells the polyphosphate bodies develop intrathylakoidally (Figure 96). These polyphosphate bodies vary in diameter from about 10 to 150 nm. Numerous polyphosphate bodies often develop within one expanded thylakoid as three may be seen in one thin section (Figure 96).





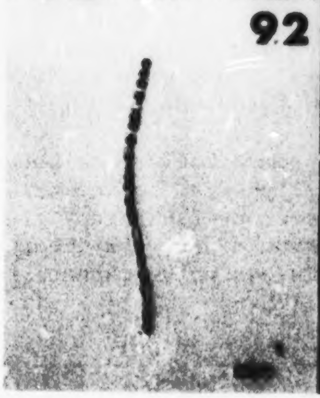


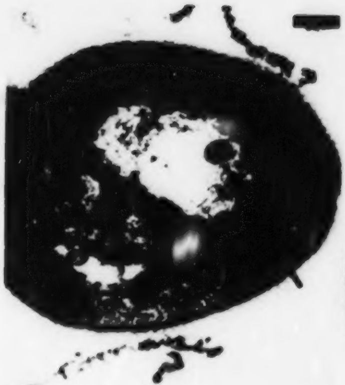
Figure 89. Rapid uptake cell showing an elongate polyhedral body (Pb). Glut-OsO_4 fixation.

Figure 90. Portion of a rapid uptake cell showing an area of medium electron density in a polyhedral body (Pb). OsO_4 fixation.

Figure 91. Portion of a rapid uptake cell showing a polyphosphate body in a polyhedral body (Pb).

Figure 92. Filament from rapid uptake culture which has been stained to show the polyphosphate bodies. Note the various sizes and morphologies of the bodies. Bar marker equal 5 µm.

Figure 93. Rapid uptake cell showing a large medium electron-dense area which has developed in a nucleoplasmic area. Note the electron-transparent areas (arrows) in which polyphosphate bodies will develop. OsO_4 fixation.



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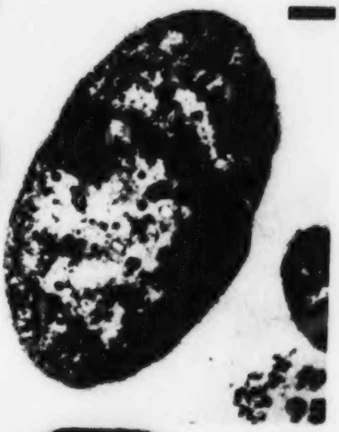

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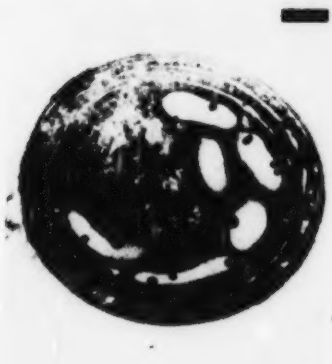

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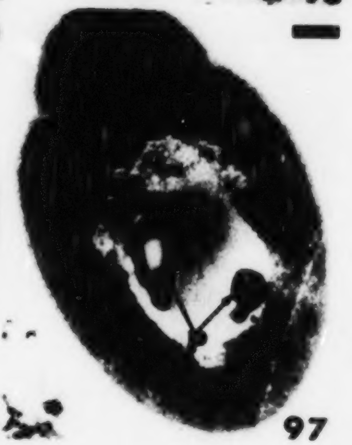

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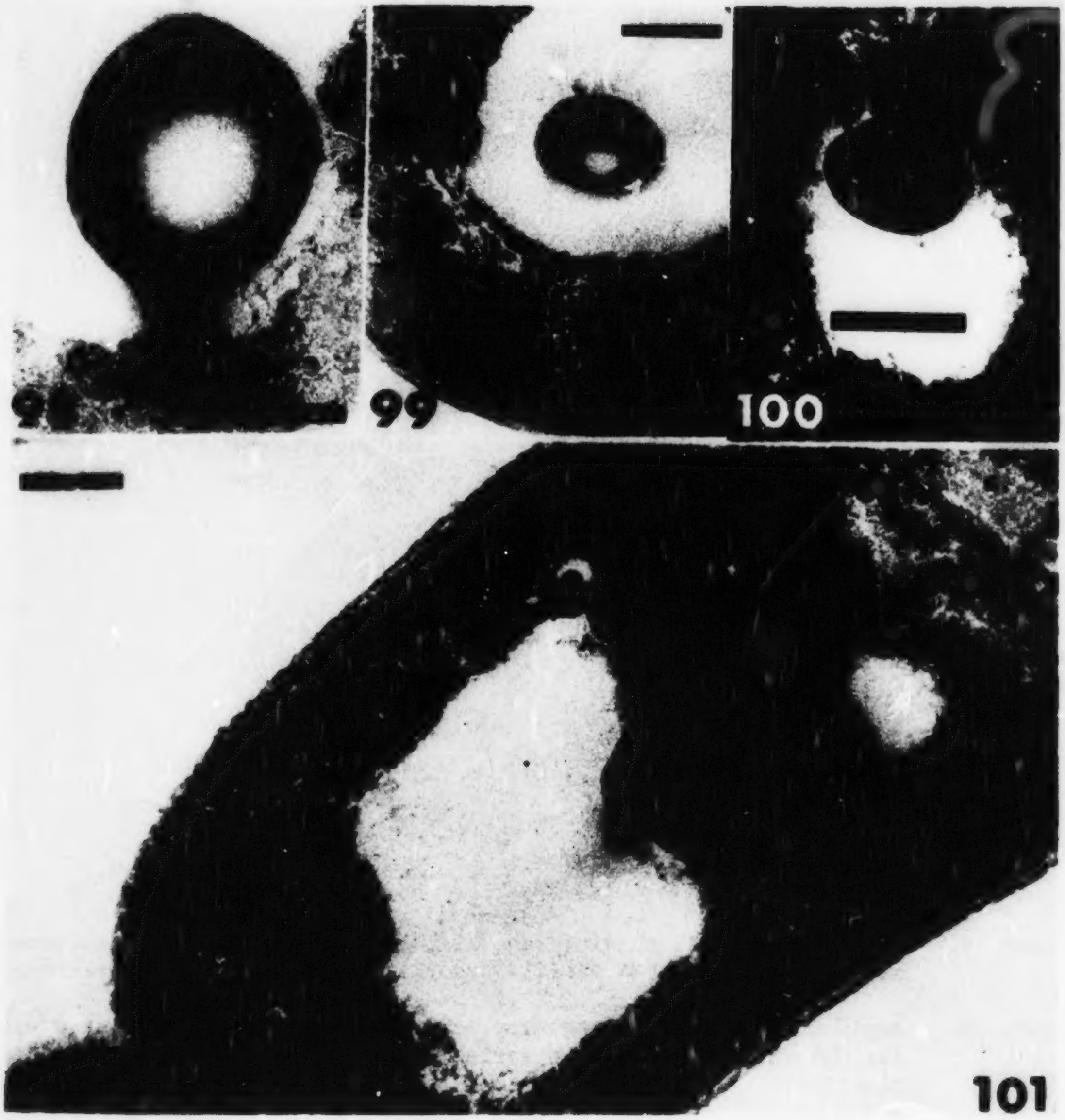
Figure 94. Rapid uptake cell showing several polyphosphate bodies, one (arrow) has many apparent DNA fibrils associated with it. OsO_4 fixation.

Figure 95. Rapid uptake cell showing many polyphosphate bodies associated with the DNA fibrils. Note the variation in their morphology. OsO_4 fixation.

Figure 96. Rapid uptake cell showing polyphosphate bodies which have developed intrathylakoidally. Glut-OsO_4 fixation.

Figure 97. Rapid uptake cell showing two large polyphosphate bodies (P) which have developed in an area of medium electron density OsO_4 fixation.



Figures 98-101. Rapid uptake cells showing morphological variation of polyphosphate bodies which have developed in areas of medium electron density. OsO_4 fixation.

In a very few cells polyphosphate bodies are seen in the ribosomal areas as previously described in P. boryanum (Jensen, 1969).

Few lipids and cyanophycin granules are seen in the cells during rapid uptake.

ENERGY DISPERSIVE X-RAY ANALYSIS

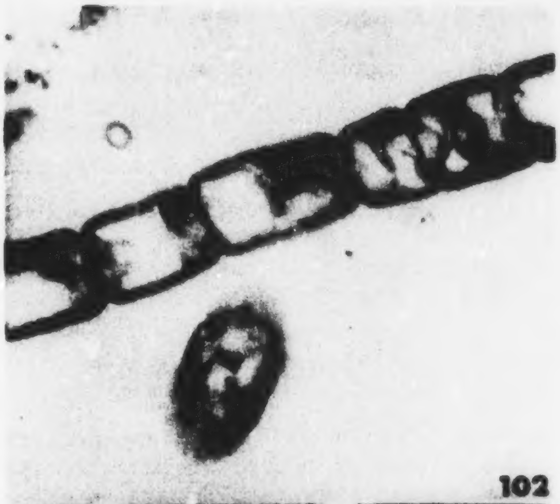
Analysis was performed in all cases after morphological identification of the polyphosphate bodies in the transmission mode of the scanning electron microscope (STEM). Typical polyphosphate bodies are shown in Figures 102 and 103. The results obtained with the various fixation and embedding procedures are as follows:

Osmium Fixation

Glutaraldehyde fixation does not yield the best preservation of morphological integrity in prokaryotes, hence they are usually fixed with osmium tetroxide. The presence of osmium, however, poses a problem in X-ray analysis because the osmium M spectral line (1.978 keV) cannot be resolved from the K spectral lines (K^{a}, 2.015 and K^{B}, 2.016 KeV). The limitation to resolution of these spectral lines, which are 28 ev apart, is the detector, which has a resolution of 152 ev. In all cases, this osmium contribution to the phosphorus peak results in a shoulder making analysis or semi-quantitative estimates difficult (Figures 104 and 105). This interference was somewhat reduced by decreasing the range of the X-ray analysis to 10 ev/channel. Other components normally encountered in the analysis of polyphosphate bodies were chlorine (K^{a} at 3.691 keV), Figures 104 and 105. Chlorine is a common component of epoxy resins and was found in the analysis of the embedding medium with no cells present (Table 7).

Glutaraldehyde Fixation

Glutaraldehyde fixation without post-fixation in OsO_4 was chosen as an alternate method of preserving the algae in an attempt to avoid osmium contribution to the phosphorus peak during X-ray energy dispersive analysis. Although this method does not provide the best image for routine electron microscope studies, it does provide sufficient preservation of the cells to allow identification of the constituents and X-ray analysis of the inclusions. Polyphosphate bodies are somewhat less dense and more difficult to identify with this fixation (Figure 103). However, analysis with this method of fixation results in a more distinct phosphorus peak without osmium interference (Figures 106 and 107). The other components present are calcium, and traces of chlorine (Figures 106 and 107). The P/B ratio of chlorine in polyphosphate bodies is significantly lower than in the cytoplasm due to the lack of permeability of epoxy resins into the bodies as well as the greater X-ray emission characteristics of phosphorus and calcium compared to organic constituents of the cytoplasm. Analyses of material fixed with either glutaraldehyde or OsO_4 are qualitatively similar (Table 7). Polyphosphate bodies consistently reveal a phosphorus and a calcium component. The cytoplasm of the cells (Figures 107 and 108) or the embedding medium (Figure 109) does not contain detectable amounts of these elements.



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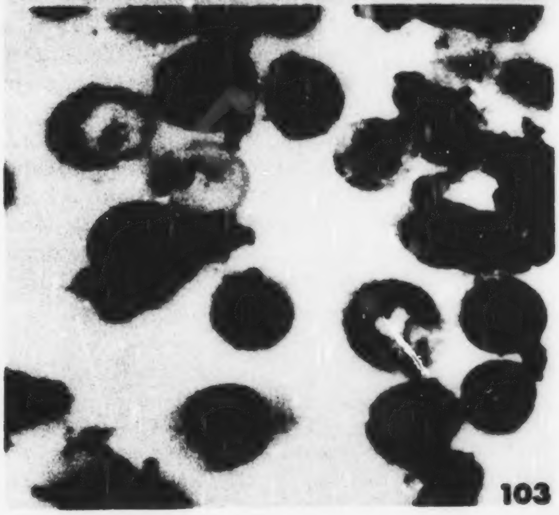

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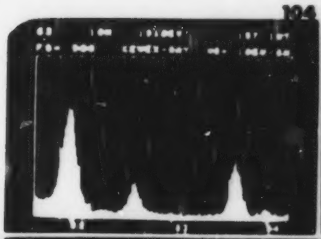
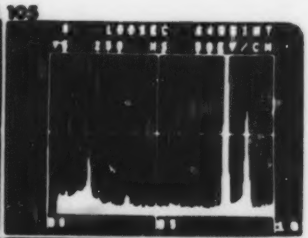
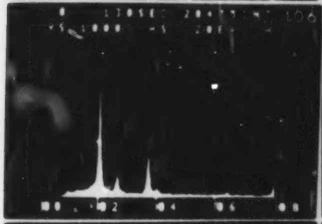
Figure 102. Scanning Transmission Electron Micrograph (STEM) of a filament of Plectonema boryanum fixed in OsO_4. Note the large, dense deposits of polyphosphate.

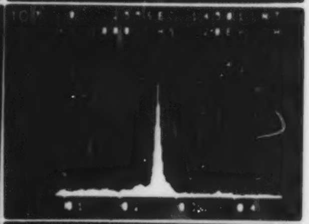
Figure 103. STEM of cells in cross section which have been fixed only in glutaraldehyde. Note the polyphosphate bodies which are not as dense as those in Figure 102.

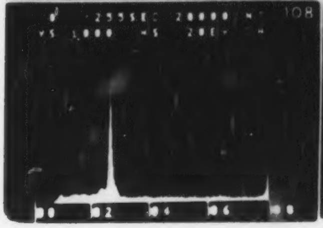
104 105











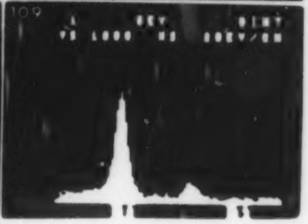


Figure 104. Analysis of a polyphosphate body fixed in osmium and embedded in Epon. The major identifiable peaks are phosphorus (2.015 and 2.016 keV), chlorine (2.622 keV) and calcium (3.691 keV). The figure shows a marker at 1010 ev to demonstrate the location of the osmium emission on the spectrum. Note also the K^B emission of calcium at 4.012 keV.

Figure 105. Analysis of a polyphosphate body fixed in osmium and embedded in Durcupan. Note the reduced separation at 50 ev/channel and the absence of chlorine in the Durcupan. Other elements present are osmium and copper.

Figure 106. Analysis of a polyphosphate body fixed in glutaraldehyde and embedded in Epon. Note the absence of a shoulder on the phosphorus peak.

Figure 107. Comparison spectra of a polyphosphate body and the adjacent cytoplasm. Dots correspond to the polyphosphate body whereas lines correspond to the cytoplasm analysis.

Figure 108. Analysis of cytoplasm fixed in glutaraldehyde.

Figure 109. Comparison spectra of a polyphosphate body and the epoxy embedding medium.

TABLE 7. P/B RATIOS. PEAK TO BACKGROUND RATIOS WERE CALCULATED BY DIVIDING THE COUNTS FROM A SINGLE CHANNEL FROM THE BACKGROUND INTO THE HIGHEST CHANNEL OF A GIVEN EMISSION PEAK

Fixation and Embedding Procedures	Area Analyzed	Element	P/B
Osmium-Epon	Poly P body	Os	2.2
		P	5.6
		Cl	2.1
		Ca	4.0
Osmium-Epon	Cytoplasm	Os	1.9
		P	2.2
		Cl	21.9
		Ca	1.1
Glutaraldehyde-Durcupan	Poly P body	Os	2.9
		P	7.6
		Cl	1.0
		Ca	2.3
Glutaraldehyde-Durcupan	Cytoplasm	Os	1.1
		P	2.6
		Cl	1.4
		Ca	1.2
Durcupan	Plastic without cells	Os	1.1
		P	1.6
		Cl	1.6
		Ca	.9

SECTION VIII

DISCUSSION

GROWTH CURVES, PHOSPHATE DEPLETION, AND MINIMUM CELLULAR PHOSPHATE LEVELS

The growth curves of *Plectonema boryanum* and *Oscillatoria tenuis* all demonstrate an increase in dry weight on the first day after transfer into fresh medium, regardless of phosphate concentration in the medium. The increase in dry weight is also accompanied by a corresponding decrease in phosphate concentration of the external medium (Figures 2-15). This increase in dry weight and depletion of phosphate from the external medium is probably due to an uptake phenomenon. Transfers of cultures were routinely made from cultures which were at least 2 months old. Analysis of the total phosphate content of cells as a function of culture age revealed that at this age, cultures had already been reduced in phosphorus content to 4 μg P per mg dry weight algae (Figure 17). Studies correlating dry weight analysis to cell number revealed that for all cultures, 1 mg of dry weight algae corresponded to 5.5 x 10^6 cells. Thus, the total phosphorus content of a 2 month old culture would equal 0.73 μg P per 10^6 cells.

Similar analysis of normal, 5 day starved, and 4 hour rapid uptake cultures yield the following information: Normal cultures - 1.5 μg P/10^6 cells; Starved cultures - 0.76 ug P/10^6 cells; and, Uptake cultures - 14.2 μg P/10^6 cells. It can be seen that the total phosphorus content of a two month old culture is slightly lower than that of a culture starved for 5 days. Normal culturing of the algae also leads to depletion of phosphorus reserves, but over a much longer period of time. Thus, transfer of these phosphate depleted cells in reality, sets the stage for a rapid uptake of orthophosphate.

These results for internal phosphorus concentration are similar to those reported for other organisms. Mackereth (1953) reported that the limiting phosphorus concentration of *Asterionella formosa* was about 0.06 μg P/10^6 cells. This diatom could also store phosphorus in concentrations as high as 7 μg P/10^6 cells. The marine diatom *Phaeodactylum tricornutum* was found by Kuenzler and Ketchum (1962) to contain phosphorus concentrations of 2.1 μg P/10^6 cells at its highest levels, and 0.06 μg P/10^6 cells at its lowest levels. Kholy (1956) reported that the minimum value below which growth did not occur in *Chlorella* was 0.1 μg P/10^6 cells. The maximum level of phosphorus in the cells was 1.5 μg P/10^6 cells. This value did not increase further when the starved algae were reinoculated into higher phosphate concentrations.

The values reported in the literature are similar to those values obtained for *Plectonema boryanum* in this study. However, it would appear that *P. boryanum* contains more total phosphorus even at its lowest levels, especially when one considers that this alga is much smaller in size than the diatoms or green alga utilized in these previous studies.

Table 2 indicates that there is an appreciable increase in dry weight of the algae during the uptake process. This increase in dry weight is also common to routine culture transfer (Figures 2-15). These results also lend support to the occurrence of the uptake phenomenon associated with transfer of algae to fresh medium during routine culture.

PHYSICAL PARAMETERS AFFECTING UPTAKE RATES

Light has been demonstrated to stimulate uptake of phosphate in many organisms (Talpasayi, 1962; Kanai and Simonis, 1968; Fitzgerald, 1970). Dark assimilation of phosphate has also been reported (Kanai, Miyachi, and Miyachi, 1963; Batterton and Van Baalen, 1968; and Overbeck, 1962). P. boryanum appears to be an organism which requires light for maximal uptake of phosphate (Figure 23). The increase in total cell phosphorus is directly related to the light intensity available. Higher light intensities during the uptake period result in higher cell phosphorus levels, up to 18.9 ug P/10^6 cells at 2000 ft-candles. Preillumination of cultures of P. boryanum during the starvation period and subsequent inoculation into a phosphate-containing medium in the dark resulted in uptake, but at greatly reduced levels. These results are unlike those reported by Simonis and Urbach (1963) who reported dark uptake in preilluminated cultures of Ankistrodesmus braunii and Anacystis nidulans (Simonis et al, 1974).

The effect of light intensity on the uptake process suggests several possibilities. Energy is required for phosphate uptake to occur. This energy can be supplied by photosynthesis, respiration, or a combination of both processes. If photosynthesis alone were involved, one would expect results such as those presented in Figure 23. ATP produced during photophosphorylation would supply the energy required for uptake. Another possible source of energy would be the metabolism of products of photosynthesis. If this were true, then inhibition of metabolic pathways involved in utilization of photosynthetic products would result in either complete or partial inhibition of the uptake process. A third alternative is the metabolism of storage materials in the cell through respiration. In this case, one would expect increased uptake with increasing light intensity, and also phosphate uptake in the dark with the metabolism of storage materials.

Figure 23 demonstrates that increasing light intensity does accelerate phosphate uptake, but there is virtually no uptake in the dark. Figure 27 illustrates the effects of several metabolic inhibitors on the phosphate uptake process. These results indicate that phosphate uptake requires a supply of metabolic energy. If metabolism of the products of photosynthesis were not involved as an energy source, one would expect no effect exerted by these inhibitors. This does not appear to be the case. Increasing light intensity most likely increases the supply of sugars to be metabolized by the alga. Some dark uptake probably indicates that sugars resulting from photosynthesis during the starvation period were probably metabolized in the dark, but no further production was possible. It is also likely that there are no other storage forms of sugar present in the alga which could readily be available as an energy source.

It is a well known fact that the optimal temperature for photosynthesis is 25 C. Temperature effects on uptake (Figure 22) and growth rates (Figure 16) indicate that although elevated temperatures (25-37 C) increased in the growth rate, they do not increase the rate of uptake.

It has been demonstrated many times that an energy source is required for phosphate uptake. Blum (1966) demonstrated that phosphate uptake by Euglena gracilis was inhibited by 2,4-dinitrophenol. Borst-Pauwels and Jager (1969) found that phosphate uptake in Saccharomyces cerevisiae was inhibited by both 0.1 mM 2,4-dinitrophenol, and 20 mM fluoride. Jungnickel (1970) found that 2,4-dinitrophenol reduced, but did not completely inhibit phosphate uptake in Candida utilis. Ullrich (1972) found that rates of polyphosphate formation were rather low when compared with the probable rates of ATP formation under various conditions of photophosphorylation in Ankistrodesmus braunii. The uptake rate of phosphate after a starvation period was higher than the rate of polyphosphate synthesis.

ION EFFECTS

Previous studies have indicated that the pH of the medium and ions effects are related to phosphorus uptake. In Ankistrodesmus braunii (Ullrich, 1972), Saccharomyces mellis (Weimberg, 1970), Dermocystidium sp. (Belsky et al., 1970) and Baker's yeast (Schmidt et al., 1946) uptake was greatest in the acidic range. However, these organisms, unlike P. boryanum, grow best in an acidic environment. In P. boryanum uptake was the greatest at pH 9. In culture media of this pH organism grew at the fastest rate. It is also interesting to note that calcium exerted the greatest effect on phosphorus uptake while in the above named organisms potassium and sodium exerted the greatest influence on uptake. This may be related to the fact that in P. boryanum the polyphosphate is associated with calcium as determined by X-ray analysis. It is also evident that ions in combination lead to greater uptake than any single ion. Calcium also seems to be necessary for rapid uptake but the uptake is enhanced by the presence of other ions either singly or in combination.

PHOSPHORUS DISTRIBUTIONS IN CELLULAR EXTRACTS

Logarithmic Cultures in Different Phosphate Concentrations

Table 5 summarizes the influence of external phosphate concentration on the distribution of phosphorus in various cell extracts. It can be seen that increasing phosphate concentrations, in general, result in higher phosphorus levels for all fractions. These results can also be correlated with the microscopy of the samples.

The ratios of acid-soluble (cold TCA extractable) phosphates to acid-insoluble (hot TCA extractable) phosphates seem to vary in many organisms. Aitchison and Butt (1973) reported that acid-insoluble polyphosphates of Chlorella vulgaris were slightly higher during logarithmic growth than acid-soluble. Baker and Schmidt (1964) found that in Chlorella pyrenoidosa, the levels of acid-insoluble polyphosphate decreased immediately prior to and during nuclear division. Krishnan et al. (1957) found that both acid-soluble and acid-insoluble polyphosphates were normally present in mycelia of Aspergillus niger. Soluble polyphosphate was present in low concentrations in new mycelia, but accumulated and reached a peak as the organism grew, after which it declined until the time of general autolysis. On the other hand, they found that insoluble polyphosphates were present in high concentrations in newly formed mycelia, and fell after about one week at these concentrations.

Plectonema boryanum, during logarithmic growth, contains more acid-soluble than acid-insoluble polyphosphates (Table 5). This is true for all phosphate concentrations tested. The standard deviations expressed in this table are a result of variability in different experiments, and not variability in the triplicate samples of a single total extraction. The increasing total cell phosphorus content is probably due to the uptake phenomenon occuring upon transfer to fresh medium. It has been demonstrated earlier that the algae, under optimal conditions, do not increase in total cell phosphorus to levels above 18.9 μg P per 10^6 cells. This maximum level occurred only at 2000 ft-candles of illumination and was not used routinely for uptake studies because the alga will not grow well at light intensities above 500 ft-candles.

In the case of growth in 1000 mg of phosphate per liter of culture medium, the total phosphorus content of the cell digest represents 6.5 μg P per 10^6 cells, a value approximately four times higher than that of the cultures grown at 10 mg PO_4 per liter. If the uptake phenomenon did occur upon transfer, one would expect increased total cell phosphorus according to the available phosphorus in the medium, but probably reduced after 14 days of culture. The phosphorus content of the total cell digests of cultures grown in 100 and 1000 mg PO_4 per liter culture medium again also indicates that the algae are able to assimilate certain levels of phosphate, and beyond a certain value, no further increase in phosphate concentration in the medium results in increased cellular phosphate levels.

Electron microscopic examination of cultures grown at various phosphate concentrations also reveals that there is increased cell death and lysis when the algae are grown at high phosphate concentrations. This might indicate that the measurements of phosphorus concentrations in the cell fractions of cells grown in higher phosphate concentrations are more unreliable, since the number of viable cells is reduced.

Starved and "Overplus" Cultures

Table 6 and Figures 58-62 demonstrate the changes in all phosphorus-containing fractions that occur when the algae are cultured in phosphate-free medium and subsequently inoculated into a medium containing phosphate. Cultures starved of phosphate for a five day period generally decrease in phosphate levels in all cell extracts examined. After the initial reduction on days 1 and 2, there was an increase, though small, on days 3 and 5. This pattern of changes in phosphorus content can be related to the fluctuations in dry weight which occur during the starvation period, and also the microscopic examination of the cultures.

There is a loss in dry weight of the cultures on days 2 and 4 of starvation. The increases in phosphorus occur when there is a reduction in dry weight. Examination of the cultures during this period reveals that there is increased cell lysis during the starvation period. Cells which do appear normal, however, often have small polyphosphate bodies. Thus it seems likely that the increasing cell lysis released phosphate back to the medium, and the surviving cells are able to utilize this phosphate for growth, even at these low phosphate levels.

The two fractions of most interest both during starvation and rapid uptake are the cold TCA extractable and hot TCA extractable phosphates. The cold TCA extract (soluble polyphosphates) decreased by about one-half during five day starvation period. The hot TCA extract (insoluble polyphosphates) initially is present in lower concentrations than the acid-soluble fraction, but is greater than the acid-soluble fraction at the end of the five day starvation period. The increase in phosphate in the acid-insoluble fraction is also greater after four hours of uptake than in the acid-soluble fraction.

These results are interesting in view of reports of starvation and uptake in other organisms. Aitchison and Butt (1973) reported that phosphate starvation of Chlorella vulgaris resulted in a general reduction of all phosphate fractions, and a 49-90 percent decrease in the acid-soluble fraction with 8 hours after starvation, and a less rapid but marked decrease in the acid-insoluble fraction. The uptake period was characterized by a rapid increase in all phosphate fractions, with the greatest over-compensation occurring in the acid-soluble fraction. The acid-insoluble fraction only recovered to the levels of cells grown in normal culture. Wiame (1949) found that in yeast, phosphate starvation resulted in a decrease in the insoluble polyphosphate fraction while the soluble polyphosphate remained constant. Phosphate uptake after an induced starvation period led to a large increase in soluble polyphosphate and no appreciable change in the insoluble polyphosphate content. The results of Aitchison and Butt (1973) using Chlorella vulgaris are comparable to those of Wiame (1949).

It has been postulated that the soluble polyphosphates (short chain length) are probably derived from insoluble polyphosphates (long chain length) by the loss of phosphate residues (Krishnan et al., 1957). If this postulate were true, one would expect greater initial increases in soluble polyphosphates during the uptake period, and a later increase in the insoluble polyphosphates due to the addition of phosphate residues to already existing primers, a reversal of the synthesis of short chain polyphosphates.

It is interesting to note that in P. boryanum, the relative levels of acid-soluble and acid-insoluble polyphosphates remain constant throughout the starvation period, even though the levels of acid-insoluble phosphate are lower in normal cells. This would suggest that the acid-soluble fraction is maintained at the expense of the acid-insoluble fraction. If synthesis occurs by addition of phosphate residues to an already existing primer (acid-soluble fraction), then the amount of acid-insoluble (long chain) phosphates is dependent upon the amount of short chain phosphates and orthophosphate. The greater increase in acid-insoluble polyphosphate at four hours of uptake is probably due to this synthesis from increased short chain primers and available orthophosphate.

Evidence exists which suggests that an enzyme, polyphosphate kinase (ATP: polyphosphate phosphotransferase, E. C. 2.7.4.1., Suzuki et al, 1972) is necessary to polymerize orthophosphate into an osmotically inert form, polyphosphate. This enzyme is synthesized during phosphate starvation (Harold and Harold, 1965; Harold, 1966). Aitchison and Butt (1973) found that in Chlorella vulgaris, polyphosphate synthesis was a consequence of the stimulation of phosphate uptake that was induced by phosphate starvation. These results, along with those of Wiame (1949) lend support to the work of Harold (1966). The synthesis of polyphosphate during rapid uptake is most likely a consequence of an energy dependent uptake of orthophosphate and subsequent polymerization by addition of phosphate residues to pre-existing primers in all organisms reported so far. The polymerization is a result of increased levels of the inducible enzyme, polyphosphate kinase. The results of experiments with P. boryanum are consistent with this hypothesis.

HYDROLYSIS AND UPTAKE OF CONDENSED PHOSPHATES

The results of hydrolysis studies of sodium tripolyphosphate are presented in Figures 63-65. From these graphs, it is apparent, especially at the higher phosphate concentrations, that hydrolysis occurs more rapidly and to a greater extent when the algae are present in the medium. At first glance, the graphs are somewhat unclear. Rapid hydrolysis, expressed as orthophosphate present in the medium, increased initially upon transfer, then decreased during the culture period at concentrations of 4.59 and 153.7 mg of phosphate present as condensed phosphate.

These results are consistent with the uptake associated with transfer to fresh medium containing orthophosphate. The algae present in the culture tubes are able to hydrolyze the condensed phosphate, but the resulting orthophosphate is subsequently assimilated by the algae which are already low in total phosphorus levels. Thus there is a reduction in the orthophosphate resulting from hydrolysis. This effect can be seen readily at the lower phosphate concentrations (Figures 63 and 64). The reduction at 1204 mg PO_4 per liter is not evident due to the high phosphate concentration (Figure 65). Hydrolysis of condensed phosphates in the absence of algae is essentially linear.

Figure 66 demonstrates that there is virtually no increase in phosphorus levels in any cell fraction when phosphate-starved algae are inoculated into medium containing condensed phosphates as the phosphorus source. This is probably due to the slow hydrolysis rate of the condensed phosphates. Maximum hydrolysis of the condensed phosphates does not begin to occur until 2 days of culture. Thus, inoculation of phosphorus deficient algae into medium containing condensed phosphates does not result in uptake of phosphate. This is due to the inability of the algae to assimilate condensed phosphates, and the low levels of orthophosphate present as a consequence of hydrolysis at this early time.

Stewart and Alexander (1971) found that there was an uptake of condensed phosphates present in detergent by the nitrogen-fixing, phosphate starved blue-green alga, Anabaena flos-aquae. However, this uptake occurred over a period of 1 day, and uptake could actually be that of orthophosphate resulting from hydrolysis of the detergent during this time period. The available literature on the utilization of condensed phosphates is scarce. However, it has been demonstrated that hydrolysis of condensed phosphates and assimilation, probably in the form of orthophosphate, occur in several green algae (Davis and Wilcomb, 1967), several blue-green algae (Davis and Wilcomb, 1968), and in the presence of various microorganisms (Clesceri and Lee, 1965a). Hydrolysis is much slower in a sterile environment (Clesceri and Lee, 1965b). Clesceri and Lee (1965b) also found a loss of phosphorus from the medium associated with the hydrolysis of condensed phosphates, probably due to uptake by microorganisms. All these results are consistent with the data presented for P. boryanum.

ELECTRON MICROSCOPY

Cells of P. boryanum grown in different phosphate concentrations exhibit a somewhat different morphology. Cells grown in either 100 or 1000 mg PO_4 per liter culture medium often possess polyphosphate bodies. They also appear to undergo lysis at a much higher frequency. On the other hand, cells grown in 1 mg PO_4 per liter are similar to cells which are grown in phosphate-free medium. They possess large areas which are of medium electron density, and large intrathylakoidal spaces. Cells grown in 10 mg PO_4 per liter possess inclusions normally found in blue-green algae, and the variations described for the other phosphate concentrations, but to a much smaller degree.

Under conditions of phosphate starvation, the cells develop morphological changes, namely, the areas of medium electron density, which later become the areas of polyphosphate body development. These areas may develop at several locations in the cell. The first of these is the nucleoplasmic area. This results in the dispersion of DNA toward the periphery of the electron lucent area and subsequent apparent association of DNA with polyphosphate bodies. The electron lucent areas are also observed in the expanded intrathylakoidal spaces, or in polyhedral bodies, with subsequent development of polyphosphate bodies in these areas. The largest polyphosphate bodies are usually located in the nucleoplasmic areas. A few small bodies are usually encountered in phosphate starved cultures. These might be correlated with the fluctuations in phosphorus content of cellular extracts during starvation and the cellular debris, probably resulting from lysis, encountered in sectioned pellets. The polyphosphate bodies could be formed from assimilation of phosphate released during the lysis. The phosphate concentration never approaches that of the external medium, and consequently the polyphosphate bodies formed are much smaller.

The mode of formation of the polyphosphate bodies in the areas of medium electron density and in the polyhedral bodies appears to be essentially the same as previously reported for the development of polyphosphate bodies in ribosomal areas in Plectonema boryanum (Jensen, 1969). Polyphosphate bodies appear to form in deposition loci, initially an area of medium electron density, and subsequently a "porous body". Electron density increased from the periphery of the body toward the center, until a completely electron dense body is formed (Jensen, 1969). This sequence was also found to be true in this study, regardless of the location of the area of medium electron density.

Thus, in P. boryanum grown under conditions of phosphate starvation and rapid uptake, polyphosphate bodies can develop in 5 different areas of the cell: (1) in ribosomal areas as previously described by Jensen (1969); (2) intrathylakoidally as previously described in Nostoc pruniforme (Jensen, 1968); (3) in association with strands of DNA which is similar to a method previously described by Voelz et al (1966) in Myxococcus xanthus; (4) in areas of medium electron density; and, (5) in polyhedral bodies. Voelz et al (1966) described three methods of formation of polyphosphate bodies under different environmental conditions during the "overplus phenomenon" in Myxococcus xanthus. Only the third method described here is similar. Voelz *et al* (1966) also described dense granules formed around polysaccharide inclusions and dense strands scattered in the cytoplasm.

Another location of polyphosphate body formation has been reported by Dierksheide and Pfister (1973). Under normal culture conditions, they found that in the blue-green alga, *Anacystis nidulans*, polyphosphate body formation occurred via the deposition of phosphate within the cyanophycin granule. This site of deposition was never encountered in *P. boryanum*.

Light microscopy of the cultures demonstrated that there was one apparent polyphosphate body present. Numerous smaller bodies would not be distinct at this limit of resolution. Thus it is difficult to state with any certainty that one is viewing a single polyphosphate body.

The variation in cell length and the unusual division observed under conditions of phosphate starvation is of interest. Phosphate has been suggested to act as a control factor in cell division in *Chlamydomonas reinhardti* (Lien and Knutsen, 1973). A similar type of variation in cell morphology has recently been reported in phosphoglucomutase-deficient mutants of *Bacillus licheniformis* grown under conditions of phosphate limitation (Forsberg *et al*, 1973). These mutants of *B. licheniformis* often developed septa at angles to the normally formed septa, and cell division did not always result in two equal daughter cells.

X-RAY ENERGY DISPERSIVE ANALYSIS

X-ray energy dispersive analysis of inclusions identified as polyphosphate bodies substantiates earlier physiological data. Polyphosphate bodies, examined under a variety of conditions, contain as the major components phosphorus and calcium. No magnesium is present as a major peak. This does not rule out its presence, but it can be said that it is not a major constituent of polyphosphate bodies in *P. boryanum*.

At present, there is little available information on X-ray energy dispersive analysis of polyphosphate bodies in other organisms. There are several reports of chemical identification, however, which substantiate the evidence presented here. Rosenberg (1966) found that pyrophosphate granules isolated from *Tetrahymena pyriformis* contained after ashing 48.5% phosphorus as pyrophosphate, 13.5% calcium, and 8.1% magnesium by weight. Munk and Rosenberg (1969) investigating this same organism, found that equimolar concentrations of calcium and magnesium ions were essential for the deposition of pyrophosphate granules. Isolated granules were found to be deposits of calcium magnesium pyrophosphate. Friedberg and Avigad (1968) reported that polyphosphate bodies isolated from *Micrococcus lysodeikticus* contained 18.39 µg mg per mg dry weight and 7.54 µg ca per mg dry weight. Cytoplasmic granules of *Tetrahymena pyriformis* analyzed *in situ* by electron probe analysis (Coleman *et al*, 1972) were found to contain potassium, calcium, magnesium, phosphorus, and a lipid-like material. The ratios of calcium to phosphorus and magnesium to phosphorus tended to divide the granules into two distinct classes. These reports are in some agreement with the data presented here.

It can be seen that much work remains to be done in this area. At present, no exhaustive study has been conducted previously on one organism, making a comparison of these studies with those reported in the literature difficult to interpret. The literature on phosphate metabolism is composed of a variety of studies utilizing diverse organisms as well as a variety of environmental and physiological parameters. Thus, selection of one organism to study seems essential.

The relationship between the laboratory investigations reported here and the field observations of other workers is also of interest. It has been suggested that phosphorus is one of the key elements implicated in the limiting nutrient controversy. The studies with P. boryanum indicate that cultures quite easily become phosphorus limited. When put into a variety of situations where phosphorus is available as orthophosphate, the algae can easily assimilate large quantities of phosphates and store it as both soluble (short chain) and insoluble (long chain) polyphosphates in the cellular inclusion known as a polyphosphate body. This then probably serves as a phosphorus reserve. The cells are still able to grow when phosphorus appears to be limiting in the medium.

Studies with uptake of condensed phosphates do not rule out detergents as sources of phosphate which are able to support the growth of P. boryanum. They merely indicate that condensed phosphates cannot be utilized directly, but must first be hydrolyzed to orthophosphate. The orthophosphate can then be assimilated and used for synthesis. It is most probable that algae, in their normal environment, are phosphorus deficient. Possession of a mechanism whereby phosphate entering the environment could be assimilated rapidly and stored in an osmotically inert form would enable organisms to survive in periods of low available phosphorus. Phosphorus could enter the environment, for example, through sewage, or land runoff after a rainfall. It is suggested that measurement of phosphate concentrations in natural bodies of water should not be the criterion for establishing phosphate levels which are conducive to accelerating eutrophication. A more meaningful estimation would be the total phosphorus present in the cell, since phosphorus stored in the cell as polyphosphate is capable of sustaining growth, even when there is no apparent available phosphate in the environment.

SECTION IX

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15. SUPPLEMENTARY NOTES

16. ABSTRACT : The effects of various external phosphate concentrations on physiological and cytological aspects of Plectonema boryanum have been studied. P. boryanum was found to tolerate a wide range of phosphate concentrations, from 1 to 1000 mg of phosphate per liter. Growth of the alga in these concentrations was characterized by changes in the subcellular distribution of phosphorus-containing compounds and in ultrastructural changes which were monitored by transmission electron microscopy.

Culturing the alga in phosphate-free or phosphate deficient medium led to general reductions of phosphate in all cell fractions examined, with the most dramatic decrease in both short and long chain polyphosphates. Cytologically, the phosphate starvation period was characterized by the development of areas of medium electron density, and vacuolization resulting from expansion of intrathylakoidal spaces.

Inoculation of the phosphate-starved algae into a medium containing a known amount of phosphate led to increases in all phosphorus-containing fractions, particularly the polyphosphates. Increases in both short and long chain polyphosphates were greater than an order of magnitude. The satisfaction of the "phosphorus debt" was met essentially within an hour. Examination of the cells revealed that the cells develop polyphosphate bodies in the characteristic areas of medium electron density that develop during phosphate starvation or phosphate limitation. X-ray energy dispersive analysis of the polyphosphate bodies confirmed that they are deposits consisting of two major elements, phosphorus and calcium.

17. KEY WORDS AND DOCUMENT ANALYSIS

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